UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

AMGEN INC.
Petitioner,

v.

ALEXION PHARMACEUTICALS, INC.
Patent Owner.

Case IPR2019-00740
U.S. Patent No. 9,718,880

DECLARATION OF DANIEL BAZARKO

Mail Stop **PATENT BOARD**
Patent Trial and Appeal Board
U.S. Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450

ALXN2056
IPR2019-00740

I, Daniel Bazarko, hereby declare and say as follows:

I. INTRODUCTION

1. I am over the age of twenty-one (21) years and otherwise competent to make this declaration, and if called to testify, I could and would testify about the information in this declaration.

2. This declaration is made in support of Alexion Pharmaceuticals, Inc. ("Alexion") in this proceeding before the United States Patent and Trademark Office. The information set forth in this declaration is based upon my personal knowledge, including my review of Alexion's public filings with the U.S. Securities and Exchange Commission ("SEC") reflecting U.S. sales of Soliris® (eculizumab), which were created at or near the time of the events they reflect, were created by Alexion persons and/or its advisors and consultants with knowledge, and were kept by Alexion in the course of its regularly conducted business activities and pursuant to Alexion's regular record-keeping practices.

3. I am Senior Vice President, Chief Accounting Officer at Alexion. I have been employed by Alexion since May 2013. I work at the Alexion site located at 100 College Street, New Haven, CT 06510. In my current role I am responsible for leading the global accounting, finance operations, shared services and manufacturing finance functions of Alexion. My specific job duties include

accounting, internal auditing, SEC filings, financial reporting, and financial analysis.

4. I understand that Alexion markets Soliris® in the United States, as well as outside the United States. I understand that the conditions for which Soliris® currently has FDA approval include (1) "Paroxysmal Nocturnal Hemoglobinuria" ("PNH"), (2) "Atypical Hemolytic Uremic Syndrome ("aHUS"), (3) "Generalized Myasthenia Gravis" ("gMG"), and (4) "Neuromyelitis Optica Spectrum Disorder" ("NMOSD"). (*See* ALXN2053 at 1, 3-4.)

5. I understand that Soliris® was approved by the FDA on March 16, 2007, for "the treatment of patients with paroxysmal nocturnal hemoglobinuria (PNH) to reduce hemolysis" ("the PNH indication"). (*See* AMG2005 at 1.) I further understand that Soliris® was launched in the United States for the PNH indication in April 2007.

6. As of September 30, 2019, Soliris® sales worldwide account for 78.4% of Alexion's total revenue. (ALXN2073 at 25.)

7. Net product sales of Soliris® in the U.S. are usually reported in Alexion's quarterly and yearly SEC filings. Net product sales here means the sum of Alexion's gross sales of Soliris® minus its net revenue adjustments (which include returns & allowances, discounts/rebates, distribution fees, and volume based arrangements). I have reviewed Alexion's annual U.S. net product sales for

Soliris® reported in Alexion's yearly SEC filings from launch in FY2007 through FY 2018, and Alexion's quarterly sales reported for Q1 through Q3 in FY 2019.

8. Appendix A attached to this Declaration lists Alexion's U.S. net product sales for Soliris® for (1) all approved indications, and (2) the PNH indication, including Alexion's annual sales from FY2007 through FY2018, and Alexion's quarterly sales for Q1 through Q3 in FY 2019.

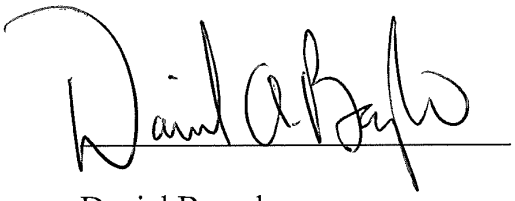
9. The documents I considered with respect to the information provided in Appendix A include: ALXN2059 (excerpts from Alexion Form 10-K for FY 2007 at Note 14, page F-34); ALXN2060 (excerpts from Alexion Form 10-K for FY 2008 at Note 17, page F-41); ALXN2061 (excerpts from Alexion Form 10-K for FY 2009 at Note 16, page F-38); ALXN2062 (excerpts from Alexion Form 10-K for FY 2010 at Note 16, page F-32); ALXN2063 (excerpts from Alexion Form 10-K for FY 2011 at Note 17, page F-35); ALXN2064 (excerpts from Alexion Form 10-K for FY 2012 at Note 15, page F-36); ALXN2065 (excerpts from Alexion Form 10-K for FY 2013 at Note 17, page F-41); ALXN2066 (excerpts from Alexion Form 10-K for FY 2014 at Note 17, page F-42); ALXN2067 (excerpts from Alexion Form 10-K for FY 2015 at F-45); ALXN2068 (excerpts from Alexion Form 10-K for FY 2016 at 58); ALXN2069 (excerpts from Alexion Form 10-K for FY 2017 at Note 18, page F-50); ALXN2070 (excerpts from Alexion Form 10-K for FY 2018 at Note 19, page F-53); ALXN2071

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(excerpts from Alexion Form 10-Q for Q1 of FY 2019 at Note 14, page 23); ALXN2072 (excerpts from Alexion Form 10-Q for Q2 of FY 2019 at Note 14, page 25); and ALXN2073 (excerpts from Alexion Form 10-Q for Q3 of FY 2019 at Note 14, page 25).

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I declare under penalty of perjury under the laws of the United States of America that the foregoing is true and correct. Executed this 22nd day of November 2019 in New Haven, Connecticut.



Daniel Bazarko

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Appendix A

Year/Quarter	U.S. net product sales for Soliris® (total) ($M)	U.S. net product sales for Soliris® (PNH only) ($M)[1]
FY 2007	**> 45**[2]	**> 45**
FY 2008	**113.2**	**113.2**
FY 2009	**159.8**	**159.8**
FY 2010	**205.8**	**> 200**
FY 2011	**263.4**	**> 200**
FY 2012	**400.5**	**> 200**
FY2013	**561.4**	**> 250**
FY2014	**730.1**	**> 350**
FY2015	**> 800**[3]	**> 400**
FY2016	**> 1,000**	**> 450**
FY2017	**1,235.0**	**> 500**
FY2018	**1,588.4**	**> 500**
Q1 2019	**463.7**	**> 100**
Q2 2019	**496.3**	**> 100**
Q3 2019	**496.8**	**> 75**

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1 Although Alexion has not typically disclosed U.S. net product sales for Soliris® ***in PNH only***, based on my role at Alexion, my knowledge of when other indications for Soliris® received FDA approval, and my knowledge of when other products for treating PNH later became available in the U.S., I am able to provide the estimates of U.S. net product sales for Soliris® in PNH shown in the last column of Appendix A.

2 Alexion did not precisely report U.S. net product sales for Soliris® in its 10-K filing with the Securities and Exchange Commission in 2007. Based on my role at Alexion and my knowledge of the initial sales figures for Soliris® in the U.S., I estimate that the U.S. net product sales for Soliris® were at least greater than $45 million for 2007.

3 Although Alexion did not expressly break down Soliris® net product sales by region (U.S., EU, etc.) for 2015 and 2016, based on my role at Alexion and my familiarity with the sales figures in addition to the publicly available sales data, I am able to estimate that U.S. net product sales for Soliris® in these years were at least greater than about $800 million and $1 billion for 2015 and 2016, respectively.

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

AMGEN INC.
Petitioner,

v.

ALEXION PHARMACEUTICALS, INC.
Patent Owner.

Case IPR2019-00739
U.S. Patent No. 9,725,504

DECLARATION OF MICHEL C. NUSSENZWEIG, M.D., Ph.D.

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Patent Trial and Appeal Board
U.S. Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450

I, Michel C. Nussenzweig, M.D., Ph.D., hereby declare and say as follows:

I. INTRODUCTION

1. I am over the age of eighteen (18) years and otherwise competent to make this declaration. If called to testify in the above-captioned matter, I could and would testify about the information in this declaration.

2. I was asked to consult in this proceeding regarding the sequencing of genetic material the hybridoma with ATCC designation HB-11625 ("hybridoma HB-11625"), maintained by ATCC, corresponding to the murine antibody sequences, to determine the sequence of the mouse monoclonal antibody "5G1.1." I understand 5G1.1 is the antibody produced from hybridoma HB-11625. (ALXN2074.)

3. This Declaration sets forth my experience, and my review and analysis of the sequencing of genetic material from hybridoma HB-11625 by the laboratory GenScript Inc. located in Piscataway, New Jersey.

4. I am being compensated at my normal rate of $ 1000/hr for the time consulting on this matter. No part of my compensation is dependent on the outcome of this proceeding, and I have no other interest in this proceeding.

II. EXPERIENCE AND QUALIFICATIONS

5. I am a distinguished Zanvil A. Cohn and Ralph Steinman Professor at The Rockefeller University, and head of the Laboratory of Molecular Immunology at The Rockefeller University and a Howard Hughes Medical Institute investigator.

6. My educational background includes me graduating summa cum laude with a B.A. degree from New York University College of Arts and Sciences in 1975. In 1981 and 1982, I received my Ph.D. in cellular immunology and M.D., respectively, from The Rockefeller University and New York University School of Medicine.

7. After I earned my M.D., I went on to complete an internship and residency in internal medicine, and a clinical fellowship in infectious diseases at Massachusetts General Hospital in 1985. From 1986 to 1989, I was a postdoctoral fellow in genetics in the Harvard Medical School laboratory of Philip Leder.

8. After I completed a postdoctoral fellowship, I became an Assistant Professor at The Rockefeller University in 1990, and I became an Associate Professor in 1994, and then a Professor and Senior Physician in 1996.

9. I have received numerous honors and awards, including the Meritorious Career Award from the American Association of Immunologists–Huang Foundation (2004); the Lee C. Howley Sr. Prize for Arthritis Research, (2008); Brazilian Diaspora Award (2015); Rabbi Shai Shacknai Memorial Prize in

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Immunology and Cancer Research (2016); Robert Koch Award (2016); AAI-BioLegend Herzenberg Award (2017); Sanofi-Institut Pasteur Award (2017).

10. I am currently a member of numerous honorary and professional societies including the U.S. National Academy of Medicine, the U.S. National Academy of Sciences, the American Academy of Arts and Sciences, and the Brazilian Academy of Sciences; the American Association of Immunologists; the American Medical Association; the New York Academy of Sciences; the American Society for clinical investigation and the American Society for Microbiology.

11. I have published more than 300 original scholarly works as peer-reviewed journals, chapters, reviews and books in the field of immunology. My current research focuses on molecular aspects of the body's innate and adaptive immune responses, and the physiological function and development of dendritic cells.

12. I am the Editor of the Journal of Immunological Methods, and the co-Chair of the Editorial Board of the Journal of Experimental Medicine.

13. A further description of my background, awards, and qualifications can be found in my curriculum vitae attached as an appendix to this declaration. My curriculum vitae is provided in ALXN2027, and attached here as **Appendix A**.

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III. SUMMARY OF OPINIONS

14. I have reviewed the Final Report, Antibody Full Length Sequencing of Hybridoma 5G1.1 T175 ("Sequencing Final Report") (ALXN2075) and GenScript's Sequencing Record (ALXN2076) created by GenScript. I also reviewed the proposed sequencing protocol prior to the sequencing (ALXN2087). The documents I reviewed in providing this declaration are listed in the attached **Appendix B**.

15. In my opinion, the sequencing performed by GenScript reported in the Sequencing Final Report was performed by appropriate methods, and the results reported in the Sequencing Final Report are reliable and accurate.

16. The full sequence of 5G1.1, a mouse antibody, from HB-11625 is accurately listed on pages 2-3 of ALXN2075.

17. The method used for sequencing was as follows:

a. I understand based on records I reviewed, specifically GenScript's Sequencing Record (ALXN2076), a GenScript chain of custody log ("Chain of Custody Log") (ALXN2077), and ATCC Packing Slip (ALXN2078), that ATCC shipped five vials of hybridoma HB-11625 to GenScript on Thursday, November 7, 2019, and that these were received by GenScript at their facility in New Jersey on Friday, November 8, 2019. (ALXN2078; ALXN2077.) The vials

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were shipped on dry ice, and were received at GenScript by Alan Chau. (ALXN2077.)

b. I understand that on Friday, November 8, 2019, GenScript shipped the samples from their facility in New Jersey to their facility in Nanjing, China, where the sequencing was performed. (ALXN2077.) The cells were shipped by Cindy Wu at GenScript's New Jersey facility and were shipped in a dry shipper that was maintained at -80°C for the duration of the shipment. (ALXN2077.) The cells were received by Tu Huamin at GenScript's facility in Nanjing, China on Tuesday, November 12, 2019, local time. (ALXN2077.)

c. On receipt at GenScript's facility in Nanjing, China, the cells were transferred to YuLing Li, with GenScript's antibody drug discovery department, and stored in a -80°C freezer. (ALXN2077.)

d. Per GenScript's Sequencing Record, hybridoma sequencing on one of the vials of hybridoma HB-11625 obtained from ATCC was performed by YuLing Li at GenScript's facility in Nanjing China from November 13, 2019 through November 15, 2019. (ALXN2076 at 1-4.)

e. According to GenScript's Sequencing Record (ALXN2076) and Final Sequencing Report (ALXN2075), GenScript first extracted RNA from hybridoma HB-11625, and performed reverse transcription to obtain cDNA from the hybridoma cells corresponding to a murine antibody. They then used PCR to

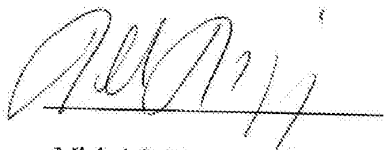
amplify fragments of cDNA corresponding to the murine antibody heavy and light chains. These were cloned into sequencing vectors. Bacteria were transformed with the sequencing vectors containing the murine antibody cDNA inserts, and DNA obtained from the resulting bacterial colonies was sequenced using PCR to identify inserts corresponding to the antibody light and heavy chains and determine the corresponding sequences.

f. The resulting heavy and light chain sequences of the murine (mouse) antibody produced by HB-11625 are provided in the Sequencing Final Report (ALXN2075). Five clones (*i.e.*, cells from five different bacterial colonies) were sequenced for each of the light and heavy chains, to confirm the accuracy of the sequencing. (ALXN2075 at 4.)

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I declare under penalty of perjury that the foregoing is true and correct.

Executed this 21st day of November 2019 in the State of New York.



Michel C. Nussenzweig, M.D., Ph.D.

Appendix A

CURRICULUM VITAE

Name: Michel C. Nussenzweig

Date of Birth: February 10, 1955

Education:

1975	B.A. - New York University College of Arts and Sciences
1981	Ph.D. - The Rockefeller University
1982	M.D. - New York University School of Medicine

Clinical Training:

1982-1985	Intern & Resident, Internal Medicine Massachusetts General Hospital
1984-1985	Clinical Fellow, Infectious Diseases Massachusetts General Hospital

Postdoctoral Training:

1986-1989	Harvard Medical School, Department of Genetics

Professional Appointments

1990-1996	Assistant & Associate Professor, The Rockefeller University
1990-1999	Assistant & Associate Investigator, Howard Hughes Medical Institute
1996-present	Professor & Senior Physician, The Rockefeller University
1999-present	Investigator, Howard Hughes Medical Institute
2000-2013	Sherman Fairchild Professor, The Rockefeller University
2013-	Zanvil A. Cohn & Ralph M. Steinman Professor, The Rockefeller University

Honors & Awards

Summa Cum Laude, New York University College of Arts and Sciences - 1975; Phi Beta Kappa, New York University College of Arts and Sciences - 1975; Alpha Omega Alpha, New York University Medical School - 1982; Bertram M. Gesner Memorial Research Award, New York University School of Medicine – 1982; Elected Member American Society of Clinical Investigators – 1997; Solomon A. Berson Award for Basic Science – 2003; American Association of Immunologists Huang Foundation Meritorious Career Award – 2004; Elected Fellow of the American Academy of Arts & Sciences – 2007; Benacerraf Lecture, Harvard Medical School - 2007; Heidelberger-Kabat Lecture, Columbia University - 2008; Lee C. Howley Sr. Prize for Arthritis Research - 2008; Elected Member in the Institute of Medicine of the National Academies – 2009; Elected Member of the National Academy of Sciences USA – 2011; Elected Member of the Brazilian Academy of Sciences – 2011; Nobel Award Lecture for Dr. Ralph Steinman, Nobel Foundation – 2011; Elected Member of Association of American Physicians – 2012; Elected to Practitioners Society of New York – 2014; Landsteiner Lecture, Vienna – 2014; Brazil Diaspora Prize 2015; Georges Kohler Lecture, Freiburg – 2015; Elected to the Spanish Royal Academy-2016; Rabbi Shai Shacknal Prize-2016; Dan H. Campbell Memorial Lecture, Asilomar – 2016; Robert Koch Prize, Berlin – 2016; Hare Memorial Lecture – 2016; Hertzenberg

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Prize, American Association of Immunology – 2017; Sanofi-Institut Pasteur Award, Paris – 2017; Amos Memorial Lecture – 2018; Charles Gould Easton Lecture – Toronto 2018; Melvin Cohn Lecture and Award – La Jolla 2019

Teaching:
Immunology, Course Organizer

Institutional:
Member Hospital GCRC Scientific Advisory Committee 1996-2002
Chair, Hospital Seminar Committee 1997-1998
Elected Senior Faculty Representative Academic Council 1999-2001
Member, Institutional Review Board for Biohazards, Radioisotopes, Toxic Chemicals, and Carcinogens 1999-2001
Chair, Animal Care and Use Committee 2000-2011
Member, Immunology Search Committee 2001
Member, Infectious Disease Search Committee 2001
Member, Virology Search Committee 2002
Faculty Advisory Committee for Presidential Search 2002
Member President's Core Planning Group 2004-2005
Member Transgenic Facility Coordinating Committee 2004-
Member Rockefeller University Faculty Search Committee 2005-
Member Rockefeller University Academic Council 2009-2012
Member, Presidential Search Committee 2010 and 2016
President, Rockefeller University Academic Senate 2011-2012
Member, Animal Care and Use Committee 2012-
Director, Christopher H. Browne Center for Immunology and Immune Diseases 2012-
Mid Career Hiring Committee 2012-2016
Chair University Lecture Committee 2013-2015

National and International:
Arthritis Foundation Molecular Immunology study section 1993-1996
NIH Immunobiology Study Section Ad Hoc reviewer 1998-999
NIH NIAID Council Ad Hoc 1998
Organizer, Keystone Symposium on Dendritic Cells 1998
NIH ALY Study Section Ad Hoc Reviewer, 1999
Organizer, Keystone Symposium on B Cells 1999
External Reviewer LMGD NICHD 2000
Damon Runyon Cancer Research Fund Review Committee 2000-2002
American Association of Immunologists Program Committee 2000-2003
March of Dimes Review Committee 1999-2005
NIH ALY Study Section 2001-2006
Organizer, Keystone Symposium on B Cell Biology 2003
The Harvey Society, Council Member 2004-2007
Human Frontiers of Science Review Board 2004-2007
North Shore Hospital Scientific Advisory Board 2004

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Chair, NIH ALY Study Section 2005-2006
Member, AAI Awards Committee 2005-2008
NIH NIAID B Cell Immunology for Protective HIV-1 Vaccines Ad Hoc reviewer 2007
Lupus Research Institute Scientific Advisory Board, Co-chair 2008 – 2012
Global HIV Vaccine Enterprise Science Advisory Committee 2008
Member, Pasteur Institute Scientific Council 2008-2016
NIH NIAID AIDS Vaccine Research Subcommittee Member 2009-2012
Scripps Korea Antibody Institute Scientific Advisory Board 2009
Member, Int'l. Program Committee for the 14th International Congress of Immunology (ICI) Osaka 2010
The Henry Kunkel Society, Secretary 2011-2014
Harvard's Children's Hospital Boston, Scientific Advisory Committee 2012-2017
Pew Foundation Scientific Advisory Board for Latin America 2012-2018
Member, Congress Committee for the 15th International Congress of Immunology (ICI) Rome 2013
Organizer, Cold Spring Harbor Symposium on Quantitative Biology—Immunity & Tolerance 2013
Organizer, Keystone Symposium on B Cell Biology 2014
Member, Research Board of the Austrian Academy of Sciences 2018

Editorial:

1996-Present	Editor, The Journal of Experimental Medicine
1999-Present	Editor, The Journal of Immunological Methods
2000-Present	Transmitting Editor, International Immunology
2002-Present	Advisory Editor, Nature Reviews Immunology
2006-Present	Editorial Committee, Annual Review of Immunology

Consultant:

Abgenix, Fremont, CA	Scientific Advisory Board	1998-2003
Zycos, Lexington MA	Scientific Advisory Board	2001-2003
Trubion Seattle WA	Scientific Advisory Board	2004-2009
Celldex, NJ	Scientific Advisory Board	2005-Present
Frontier Bio, China	Scientific Advisroy Board	2017-Present

Professional Memberships:
American Association of Immunologists
American Medical Association
The New York Academy of Sciences
Kunkel Society
Harvey Society
American Society for Clinical Investigation
American Society for Microbiology

Invited Chapters or Reviews

1. Steinman, R.M., Witmer, M.D., **Nussenzweig, M.C.,** Chen, L.L. & Cohn, Z.A. Dendritic Cells: an important new cell type in the mixed leukocyte reaction. In: Kaplan JG, ed. Proc. of the 13th International Leukocyte Culture conference. *Elsevier/North Holland Publ.* Co. 273 (1979)

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4. Shimizu, A., Kinashi, T., **Nussenzweig, M.C.,** Tataunobu-Ryushin, M., Leder, P., & Honjo, T. Molecular mechanism for immuno-globulin double-isotope expression. *Cold Spring Harbor Symposia on Quantitative Biology* LIV 175 (1989)

5. Papavasiliou, F., Jankovic, M., Gong, S. & **Nussenzweig, M.C.** Control of immunoglobulin ene rearrangements in developing B cells, review. *Curr. Opin. Immunol.* 9:233-244 (1997)

6. **Nussenzweig, M.C.** Immune responses:Tails to teach a B cell. *Curr. Biology*. 7(6):R355-357 (1997)

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10. Nagaoka, H., Yu, W. & **Nussenzweig, M.C**. Regulation of RAG expression in developing lymphocytes. *Curr. Opin. Immunol.* 12:187-190 (2000)

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14. Steinman, R.M., Hawiger, D., Liu, K., Bonifaz, L., Bonnyay, D., Mahnke, K., Iyoda, T., Ravetch, J., Dhodapkar, M., Inaba, K. & **Nussenzweig, M.C.** Dendritic cell function in vivo during the steady state: a role in peripheral tolerance. *Annals NYAS.* 987:15-25 (2003)

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18. von Boehmer, H. & **Nussenzweig, M.C.** The manipulation of immunity. *EMBO J.* 5(8):766-771 (2004)

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29. Yurasov, S. & **Nussenzweig, M.C**. Regulation of autoreactive antibodies. *Curr Opin. Rheumato.* 19(5):421-426 (2007)

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Appendix B

APPENDIX B

Exh. No.	Description
ALXN2027	*Curriculum Vitae* of Michel C. Nussenzweig, M.D., Ph.D.
ALXN2074	ATCC Product Sheet for HB-11625
ALXN2075	GenScript, "Final Report – Antibody Full Length Sequencing of Hybridoma 5G1.1 T175," Order Number: U856UEK140-1 (Nov. 15, 2019)
ALXN2076	GenScript Sequencing Record
ALXN2077	Chain of Custody Log
ALXN2078	ATCC Packing Slip



Deposition of:

Joseph P. Balthasar , Ph.D.

October 23, 2019

In the Matter of:

Amgen vs. Alexion

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UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

AMGEN, INC.	
Petitioner	IPR No.
vs.	2019-00740
ALEXION PHARMACEUTICALS, INC.	
Patent Owner	

DEPOSITION OF JOSEPH PATRICK BALTHASAR, Ph.D.

Washington, D.C.

October 23, 2019

REPORTED BY: Tina Alfaro, RPR, CRR, RMR

Videotaped deposition of JOSEPH PATRICK BALTHASAR, Ph.D., held at the offices of:

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Washington, D.C. 20005

Taken pursuant to notice before Tina M. Alfaro, a Notary Public within and for the District of Columbia.

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Angie Mah (Amgen)

Paula Fritsch (Amgen)

I N D E X

EXAMINATION

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EXHIBITS

(None marked)

THE VIDEOGRAPHER: Good morning. We are going on the record at 9:36 a.m. on October 23rd, 2019.

Please note the microphones are sensitive and may pick up whispering, private conversations and cellular interference. Please turn off all cell phones and place them away from the microphones as they can interfere with the deposition audio. Audio and video recording will continue to take place unless all parties agree to go off the record.

This is media unit 1 of the video recorded deposition of Joseph Balthasar, Ph.D., in the matter of Amgen, Inc. v. Alexion Pharmaceuticals, Inc. filed in the United States Patent and Trademark Office, Case No. IPR 2019-00739. This deposition is being held at Sterne Kessler located at 1100 New York Avenue, Northwest, Washington, D.C.

My name is Glenn Fortner from the firm Veritext, and I'm the videographer. The court reporter is Tina Alfaro from the firm Veritext. I'm not related to any party in this action, nor am I financially interested in the outcome

Counsel and all present in the room and

everyone attending remotely will now state their appearances and affiliations for the record. If there are any objections to proceeding, please state at the time of your appearance beginning with the noticing attorney.

MR. DIAMOND: Evan Diamond of King & Spalding on behalf of Alexion.

MS. YEN: Vanessa Yen from King & Spalding on behalf of Alexion.

MS. RAHEMBA: Tara Rahemba of Alexion.

MR. FLATTMANN: Gerald Flattmann of King & Spalding on behalf of Alexion.

MS. STERLING: Debra Sterling on behalf of Amgen.

And just before we go further, this is the consolidated proceeding for the three.

MR. DIAMOND: Yeah -- yes. I -- I was going to state on the record at some point before we started I think we can agree and please confirm you agree that this deposition is -- is for the three proceedings IPR 2019-739, 740, and 741, right?

MS. STERLING: That was our understanding too.

MR. DIAMOND: Okay. Great.

MS. STERLING: Thank you.

MR. DIAMOND: Thank you.

MS. STERLING: Now we will proceed with the rest of the roll call.

MR. DIAMOND: Yeah.

MR. HOLMAN: David Holman of Sterne Kessler Goldstein & Fox on behalf of Amgen.

MS. MAH: Angie Mah of Amgen.

MS. FRITSCH: Paula Fritsch of Amgen.

THE VIDEOGRAPHER: Will the court reporter please swear in the witness.

(Witness sworn.)

WHEREUPON:

JOSEPH PATRICK BALTHASAR, Ph.D., called as a witness herein, having been first duly sworn, was examined and testified as follows:

EXAMINATION

BY MR. DIAMOND:

Q. Good morning, Dr. Balthasar.

A. Good morning.

Q. Would you please state your full name for the record.

A. Joseph Patrick Balthasar.

Q. And where do you live?

A. I live in Lancaster, New York.

THE REPORTER: I'm sorry?

THE WITNESS: Lancaster, New York.

BY MR. DIAMOND:

Q. And where do you currently work?

A. I work at the University at Buffalo.

Q. What's your -- what's your position there?

A. I'm a professor of pharmaceutical sciences. I also am currently the executive director of research initiatives for the university.

Q. Have you ever had your deposition taken before?

A. I have.

Q. And when was that?

A. Within the last couple weeks.

Q. And was that --

A. The deposition, I guess.

Q. Prior to that deposition a few weeks ago had you had your deposition taken before that?

A. No.

Q. And what kind of proceeding was -- was the one you were involved in the last couple of weeks? Was that a patent case?

A. It was a patent case, IPR proceeding.

Q. An IPR proceeding, and in that IPR proceeding were you representing the Patent Owner or the -- the Petitioner who was seeking to challenge the patent?

A. Yeah. Is that relevant? I'm sorry.

Q. I just -- if you could answer the question. Do you know -- do you know which side you were on? Were you on the patentee side or the -- or the other side?

A. Yeah. I guess I was on the Petitioner's side.

Q. Okay. And who were the -- who were the parties in this case? Who were you -- who was the party you were working for there?

A. It was Eli Lilly.

Q. Okay. And who was the party on the other side?

A. It was Teva Pharmaceuticals.

Q. Okay. And you said that was an IPR proceeding?

A. That's right.

Q. Okay. And for that case did -- did you have more than one deposition or just one?

A. There's been one deposition taken. Another will take place later on.

Q. Okay. All right. And you submitted declarations in that case in support of Eli Lilly's positions?

A. That's correct.

Q. Generally, what's the -- the technology at issue in that case?

A. Generally related to an antibody that -- an antibody product I guess.

Q. Uh-huh. Uh-huh. Is it a monoclonal antibody?

A. Yes.

Q. What does that mean?

A. What does monoclonal mean?

Q. Uh-huh.

A. It means that the antibody's derived from a single cloned population of cells.

Q. So it's a -- it's a single type of antibody?

MS. STERLING: Objection, form.

A. So it's a single type of antibody. It's an antibody that has a single defined primary amino acid sequence.

Q. Okay. And that's what a person of -- in this -- in the context of these proceedings involving Alexion and Amgen you've offered opinions

regarding who a person of ordinary skill in the art is?

A. That's right.

Q. And you've considered what a person of ordinary skill in the art would have understood regarding the patents at issue and the technology at issue in these proceedings prior to a certain date, March 15th, 2007?

A. That's correct.

Q. And the understanding you just said of a monoclonal antibody, would a person of ordinary skill in the art with respect to the patents at issue in these proceedings have had that understanding --

A. Yes.

Q. -- prior to March 15th, 2007?

A. Yes.

MR. DIAMOND: Yes, I will. Thank you for letting me know to slow down. Okay.

BY MR. DIAMOND:

Q. So I know you've had your deposition taken before, but just some quick ground rules here. I'll be asking questions and you'll be giving answers. You understand that, right?

A. Yes, I do.

Q. The court reporter is taking a record of what we say and the videographer is also recording. You understand that, right?

A. I do.

Q. Okay. And both -- we'll try to slow down a little bit so we'll make sure that everyone is able to take down what we say.

With that in mind, we'll need verbal answers. So no head nods or uh-huh. You have to say words. Okay?

A. I understand that.

Q. And your counsel at certain points during the proceeding, they may object to some of my questions. That's just for the record. You understand that you're to answer my questions unless your counsel specifically instructs you not to answer. Okay?

A. I understand.

Q. Okay. And if you don't understand any of my questions today or you need me to clarify a question, would you please let me know and -- is that -- is that okay?

A. That is, yes.

Q. And if you answer a question, I'll understand that -- that you understood the

question; is that fair?

A. That's fair.

Q. You understood that you took an oath just now to tell the truth as if you were testifying live in court or before the Patent Office, right?

A. I understand.

Q. And is there any reason you can't give me true and accurate testimony today?

A. No.

Q. If you need a break at any point today, please let me know. The only thing I ask is if there's a question that's pending that you answer the question before you take the break; is that fair?

A. That's fair.

Q. So you -- you understand that -- well, let me step back.

You're here today on behalf of Amgen, right?

A. That's correct.

Q. And you understand that Amgen has filed three inter partes review or IPR petitions with the Patent Office that challenged the patentability of -- of three patents owned by Alexion that cover the drug Soliris, right?

A. I do understand.

Q. You'll understand if I refer to these proceedings as IPR's?

A. Yes.

Q. And you understand you're testifying here today because you submitted three declarations to support Amgen's unpatentability positions in those IPR proceedings, right?

A. I understand, yes.

MR. DIAMOND: Let's -- yeah, we'll mark these. I think we'll need a stamp for these because for some reason these don't have a -- okay. So could we -- could we mark these AMG-1002 with the -- with this caption and then the other -- this is AMG-1002 with this caption and this is AMG-1002 with -- the caption actually has a typo in it. It should be 20 -- 2019.

THE REPORTER: 1002, 3, 4?

MR. DIAMOND: No. It's 1002 IPR 2019.

THE REPORTER: You have to give them exhibit numbers.

MR. DIAMOND: All right. Let's do it differently. Forget the stamps. Yeah, because it's not -- it's not written on here, but it's written on second page. So it's fine. All right.

BY MR. DIAMOND:

Q. So you've been handed three documents, Dr. Balthasar, and would you please confirm one of them is your declaration submitted in case IPR 2019-00739? Do you have that one in front of you? There's three of them. So why don't you find that one first.

A. Yes.

Q. Okay. So -- and if you turn to the second page of that document, do you see that that document was designated Exhibit AMG-1002 in the proceeding regarding U.S. Patent 9,725,504?

A. Yes, I do see AMG-1002.

Q. Okay. So we'll -- that -- that -- that will be -- you'll understand if I refer to that as AMG-1002 with respect to the '504 --

A. Yes.

Q. -- patent proceeding or the -- or IPR 2019-739?

A. Yes.

Q. Okay. Fine.

There should be another declaration in front of you and it -- it says -- it's your declaration -- I think there's a typo here. It says Case IPR 2019-00740.

A. Yes, I see that.

MR. DIAMOND: And I -- I ask counsel to confirm that -- that should be IPR 2019-00740, right?

MS. STERLING: Confirmed.

MR. DIAMOND: Okay.

BY MR. DIAMOND:

Q. So you understand, Dr. Balthasar, that -- well, if you turn to the second page of -- of this document, you see that it is Exhibit AMG-1002 in the IPR regarding U.S. Patent No. 9,718,880?

A. Yes.

Q. Do you see that?

And you'll -- you'll understand that if I refer to that as your declaration or AMG-1002 in the '880 Patent proceeding or in proceeding 2019-00740?

A. Yes.

Q. Okay. Great.

And then there's a third declaration. This is in case No. 2019-00741; do you see that?

A. Yes, I do.

Q. And that declaration that's in front of you, if you turn to the second page, you see this

is AMG-1002 in the proceeding regarding U.S. Patent No. 9,732,149?

A. I see that, yes.

Q. And you'll understand if I refer to that as your declaration regarding the '149 Patent or your -- your declaration or -- or AMG-1002 in proceeding 2019-00741?

A. Yes.

Q. Okay. Great.

Who drafted your -- your three declarations?

MS. STERLING: I'm going to object to the extent that gets to privilege. You may talk about generally the process involved in preparing your declaration, but please don't talk about any substance that was discussed between you and counsel.

A. The first drafts were prepared by counsel and we went back and forth to discuss them, and in the end I adopted these as my own. I approved completely of the declarations.

Q. Uh-huh. And prior to the first drafts being prepared by counsel -- I'm not asking for the substance of your communications, okay, but did you have conversations with counsel regarding these

proceedings before that first draft?

A. Yes.

Q. Do you know how many conversations you had with counsel before that first draft?

A. Honestly, I do not. We -- we submitted these in February. So there were a few conversations before I received the first draft. It was some time ago.

Q. Is everything in your declarations true and correct?

A. There are a couple things that I found that are typos that I'd like to be able to correct if possible.

Q. Okay. So have you -- have you yet in the context of this case submitted any corrections that you're aware of to your declarations?

A. I'm sorry. Could you repeat the question.

Q. Are you aware of whether in the context of this case before today so far you or your counsel on your behalf have submitted any corrections to your declarations?

A. I'm not aware of any corrections being submitted. These were things that I just noticed actually yesterday in going through -- you know, in preparation for today.

Q. What -- what time yesterday did you notice them?

A. It was in the afternoon. I don't remember exactly when. Yesterday afternoon.

Q. And did you let -- I'm not asking about the substance of your communication, but did you let your counsel know about those corrections?

A. Yeah. I did talk to them about it. Honestly, they're not, I don't think, of tremendous significance, but I thought that it would be good to be able to correct it since you gave me the opportunity.

Q. They're minor issues, they're not affecting the major substance of your declarations?

THE REPORTER: I'm sorry. You have to repeat.

BY MR. BALTHASAR:

Q. They're minor issues, they're not affecting the major substance of your declarations?

A. That's correct.

Q. All right. And aside from those minor issues or typos, is everything in your declaration true and correct?

A. Yes.

Q. All right. And if we're reading along and

you run into one of those typos, you'll let me know. Okay?

A. Yeah. Is it okay to just indicate it now?

Q. I'd rather we just -- I'll ask you questions, and then if we're running along and you know there happens to be a typo in something that you're reading from your declaration, just let me know.

A. Okay.

Q. We can -- we can maybe turn to it later. For now let's keep going here.

And you signed your declarations, right?

A. That's correct.

Q. You reviewed them before you signed them and you did your best to at that time confirm that everything in your declarations was true and correct, right?

A. That's correct.

Q. And you signed them under penalty of perjury, right?

A. That's correct.

Q. All right. These are -- these are stamped. Okay. These are already stamped. Thank you.

A. Thank you.

Q. So I've handed you three documents and each one is stamped AMG-1001 in the lower right-hand corner; do you see that?

A. I do.

Q. But one of the documents that you have in front of you is U.S. Patent No. 9,732,149, right?

A. Yes, that's right.

Q. And another of the documents stamped AMG-1001 that you have in front of you is U.S. Patent No. 9,725,504, right?

A. Yes, that's correct.

Q. And another of the AMG-1001 exhibits you have in front of you is U.S. Patent No. 9,718,880, right?

A. Yes, that's correct.

Q. And you understand that these three documents stamped as AMG-1001 are the three patents that are at issue in the IPR proceedings where you've submitted declarations on Amgen's behalf, right?

A. Yes, I understand.

Q. And will you understand if I refer to these individually as the -- the '149 Patent, the '504 Patent, and the '880 Patent?

A. Yes.

Q. Okay. And will you understand if I refer to them collectively, the three patents together, as the challenged patents?

A. Yes.

Q. Okay. And when did you first see the challenged patents?

A. I don't recall exactly. Probably either late 2018 or early 2019.

Q. And that -- you first saw these challenged patents in the context of your involvement in these proceedings on behalf of Amgen, right?

A. Yes, that's correct.

Q. Okay.

And did you -- did you see these patents, the challenged patents, prior to receiving the first draft of your declaration from Amgen?

A. I'm sure that I did. I don't recall exactly, but I'm sure that I would have, yeah.

Q. Did you do your best to include all of the opinions that you had regarding the patentability of -- of these three challenged patents in your three declarations in these proceedings?

MS. STERLING: Objection, relevance.

A. I don't know. That's a hard question for me to answer in a way. I'm sure I had many

opinions that were discussed back and forth. What was included in the declaration were opinions that were relevant to the challenge. And so I'm sure I had opinions that may not have been included, but the relevant ones for the patent challenge were all included within the declaration.

Q. Fair enough. If we take the '149 Patent as an example, you did your best to include all the relevant opinions that you had regarding the patentability of that patent in your declaration regarding the '149 Patent?

MS. STERLING: Objection, relevance.

A. Yeah. I would say that I'm not a trial attorney or patent lawyer, patent attorney. So I didn't have, I'd have to admit, a lot of knowledge about elements of patent law. And so it's only really through discussions with counsel that I was able to understand relevance and, you know, the intent was to include all things relevant to these patent challenges within the declarations.

Q. Let me put it this way. Did you have any opinions that you held back for later?

MS. STERLING: Objection, relevance.

A. Do you mean do I have -- did I have any relevant opinions?

Q. Yeah. Relevant -- do you have any relevant opinions -- so I'll try not to talk over you.

Did you have any relevant opinions regarding these three challenged patents that you held back for later?

A. No, there was --

MS. STERLING: Objection, relevance, scope.

A. There was no intent to hold anything back for use at a later time.

Q. All right. You're -- you're not planning on raising new arguments or evidence later on in these proceedings that -- that you could have raised in your three declarations here, right?

MS. STERLING: Objection, relevance, scope.

A. I'm not planning to, you know, bring up new information at some later point.

Q. Okay.

Could you take the '149 Patent out and if you can go to the claims of the '149 Patent. It's on the page No. 28 if you look at the page numbers that are in the center of the page at the bottom in column 39 of the patent. Are you there?

A. I am, yes.

Q. All right. So claim 1 of the '149 Patent reads "An antibody that binds C5 comprising a heavy chain consisting of Sequence ID No. 2 and a light chain consisting of Sequence ID No. 4"; do you see that?

A. I do.

Q. So this -- this claim requires an antibody that has a -- a heavy chain consisting of Sequence ID No. 2 that's identified in this patent and a light chain consisting of Sequence ID No. 4 that's defined in this patent, right?

MS. STERLING: Objection, form.

A. I do see the claim sentence that says "An antibody that binds C5 comprising a heavy chain consisting of Sequence ID No. 2 and a light chain consisting of Sequence ID No. 4."

Q. Right. So what -- you understand that one of the elements that this claim requires is a heavy chain consisting of Sequence ID No. 2 and a light chain consisting of Sequence ID No. 4, right?

A. And I'm not sure what you're getting to. I do see that this is claiming "An antibody that comprises a heavy chain consisting of Sequence ID No. 2 and a light chain consisting of Sequence ID

No. 4." I'm not sure I understand the nuance.

Q. Right. So it's -- it's a simple question. There's -- there's several elements in this claim, right, and one of the elements -- well, let's start at the beginning.

One of the elements of this claim is an antibody that binds C5, right?

A. I see that, yeah.

Q. And -- and another element of this claim is a heavy chain consisting of Sequence ID No. 2 and a light chain consisting of Sequence ID No. 4, right?

A. Yes.

Q. All right. And you understand that Sequence ID No. 2 and Sequence ID No. 4, those correspond to amino acid sequences that are defined in -- in the body of the '149 Patent, right?

A. Yes, I understand that.

Q. And those amino acid sequences for Sequence ID No. 2 and Sequence ID No. 4, those sequences are also defined in the -- in the body of the other two challenged patents in this case, the '504 Patent and the '880 Patent, right?

A. Yes, I understand.

Q. And you understand that all of the

challenged claims in these three patents require at least that that element of an antibody that has the heavy chain consisting of Sequence ID No. 2 and the light chain consisting of Sequence ID No. 4?

MS. STERLING: Objection, foundation, form.

A. Yeah. I'm not positive on that actually. Is claim 1 of '880 not requiring the antibody of that composition?

Q. Let's take a look. So you have the '880 Patent?

A. So looking back, yes, I see that it does require an antibody with the sequence ID's that are listed, yes.

Q. Right. So that the '880 Patent, all -- all of its challenged claims require an antibody comprising a -- a heavy chain consisting of Sequence ID No. 2 and a light chain consisting of Sequence ID No. 4, right?

A. Yes.

Q. And the same is true for all of the challenged claims of the '504 Patent, right?

A. Yes.

MS. STERLING: Objection, foundation.

BY MR. DIAMOND:

Q. So going -- going back to the '149 Patent, so in addition the '149 Patent requires an antibody that binds C5, right?

A. Yes.

Q. And the '504 Patent, do you have that in front of you, the claims of that?

A. Yes, I do.

Q. Do you see that all -- all of the claims in the '504 Patent, they all -- they all stem from or depend on -- on claim 1; do you see that?

A. Yes, I see that.

Q. And so you understand that all of the claims of the '504 Patent require a method of treating a patient suffering from paroxysmal nocturnal hemoglobinuria or PNH comprising administering to that patient a pharmaceutical composition comprising the antibody that we discussed, right?

A. Yes.

Q. And what is PNH?

A. PNH is a disease where complement activity leads in part to destruction of red blood cells.

Q. And what does it mean to treat PNH?

MS. STERLING: Objection to the extent it calls for a legal conclusion.

A. I would say that in the context here it's the administration to a patient a pharmaceutical composition comprising of an antibody that binds to C5 and has Sequence ID No. 2 and No. 4. So in the context I think that's relevant for '504 it's referring to administration of this type of antibody to a patient with PNH.

Q. All right. And the -- the goal of treating a patient like that is to improve their condition, right?

MS. STERLING: Objection to the extent it calls for a legal conclusion and form.

A. Yeah. It's -- I think that patients would be treated generally in a manner that might relieve symptoms or provide some type of relief, and so there would be many reasons maybe to treat a patient that might extend beyond the treatment of the primary condition. So I don't -- I don't know that I feel that treatment is something that can be defined narrowly.

Q. Okay. But broadly speaking, treatment of PNH or other conditions would involve some alleviation or attenuation or improvement of some symptoms of the condition; is that fair?

MS. STERLING: Objection to the extent it

calls for a legal conclusion and form.

A. I guess my understanding of the motivation to treat would be to provide some relief of symptoms or improvement of a condition.

Q. Okay.

And you see that claims 9 and 10 of the '504 Patent actually require some specific clinical responses to the treatment, right?

MS. STERLING: Objection, form and to the extent it calls for a legal conclusion.

A. I -- I understand from claim 9 that it's referring to a condition where administration of the antibody results in immediate and sustained decrease in mean levels of lactate dehydrogenase, LDH, and claim 10 is where the immediate decrease occurs within one week of administration of the antibody. And so in the context of the claims I feel that it's consistent with what you've said.

Q. You mean those claims -- just to make sure I understand your answer here, you agree that claims 9 and 10 of the '504 Patent are reciting -- you know, with the language that you quoted there from the claims, that they're reciting specific clinical results from the treatment?

MS. STERLING: Objection, form and to the

extent it calls for a legal conclusion.

A. So in claim 9 it -- it does refer to antibody results and gives qualification of what that means with respect to claim 9, and claim 10 goes furthers to further qualify what is meant by immediate decrease referring back to LDH as occurring within one week of administration of the antibody.

Q. And those are clinical results from treatment, right?

MS. STERLING: Objection, form and to the extent it calls for a legal conclusion.

A. So when you say "clinical results," do you mean relating to treatment of a patient?

Q. Yes.

A. Yes. So claim 1 is referring treating a patient.

Q. All right. And claims 9 and 10 refer to clinical results of treating a patient, right?

MS. STERLING: Objection, form and to the extent it calls for a legal conclusion.

A. They're dependent claims on claim 1, and so they all refer to claim 1.

Q. Yeah. And the -- the additional elements recited in claims 9 and 10 refer to specific

results of the method of treatment in patients, right?

MS. STERLING: Objection, form and to the extent it calls for a legal conclusion.

A. My understanding is that claim 9 and claim 10 are dependent claims to claim 1, and claim 1 refers to a method of treatment of a patient. And I'm -- I'm not really sure what you're getting to, but I think it's directly as stated within the claims. I understand the claims as written.

Q. Let me put it this way. So you understand that claim 9 recites certain results from the method of treating a patient, right?

A. Yeah. Claim 9 says "Wherein administration of the antibody results" and then lists those certain qualifications to the results.

Q. And those are results that would occur in the patient as a consequence of the method of treatment, right?

MS. STERLING: Objection to the extent it calls for a legal conclusion, form.

A. Yeah. So they're dependent claims. Claim 1 refers to treatment of a patient. And so I guess you're asking can I take from this that the

results relate to the patient treated, and my answer to that is yes, I -- I, you know, would read this as this refers to the patient treated.

Q. Okay. Fair enough.

If you look at the '880 Patent, claim 1 of the '880 Patent. Let me know when you're there at the claims.

A. I'm there.

Q. Okay. Claim 1 of the '880 Patent in addition to reciting the -- the specific antibody with the sequences that we discussed, it recites "A pharmaceutical composition for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH)"; do you see that element?

MS. STERLING: Objection, form.

A. I do see that element where it says "A pharmaceutical composition" and -- and moves forward from there, yes.

Q. Okay. So this -- this claim is directed to -- I guess it's different than the other two claims we've looked at so far. This is directed to a pharmaceutical composition that's -- that's suitable for -- for treating the patient who has paroxysmal nocturnal hemoglobinuria or PNH, right?

MS. STERLING: Objection, form and to the

extent it calls for a legal conclusion.

A. Yeah. I don't see any reference in claim 1 to treatment -- I'm sorry. Could you repeat your question? Maybe I didn't understand it.

Q. Yeah. So this -- this -- I'm trying to point out that this claim 1 was different than claim 1 -- well, let me rephrase that.

This claim 1 of the '880 Patent, it's directed to a pharmaceutical composition, right, that -- that could be used to treat patients who have this -- this PH -- PNH condition that we're discussing, right?

MS. STERLING: Same objections.

A. Yeah. I do see that what is claimed is a pharmaceutical composition for use in treating a patient afflicted with PNH. I do see that.

Q. Okay. So you understand it requires that the composition be -- be useful for treating that condition, right?

MS. STERLING: Objection to the extent it calls for a legal conclusion.

A. Yeah. I -- I don't know that -- that I see that requirement. So in other words, I guess this could be a composition that has these

characteristics that would be appropriate for use in treating a patient with PNH, but the -- I don't see that this is limited to treatment of PNH. In other words, the way you asked the question suggested that it would be -- might not have utility beyond that. I don't see that within --

Q. I understand. So it -- it could be a composition that would be appropriate for use in treating PNH, but also could potentially be used for treating other conditions?

MS. STERLING: Objection, form and to the extent it calls for a legal conclusion.

A. Yeah, I think that's possible.

Q. And the composition of claim 1 of the '880 Patent also has to be a "Sterile, preservative-free 300-milligram single-use dosage form comprising 30 milliliters of a 10-milligram-per-milliliter antibody solution" of the claimed antibody, right?

A. I do see that, yes.

Q. We can talk about the others later. Let's move on for now.

So I think we mentioned this earlier, but you understand that March 15th, 2007 is the priority date being used in this case?

A. Yes, I do.

Q. So you understand that's the date being used for assessing, you know, the state-of-the-art that's relevant to these patents and what is and what isn't prior art with respect to the three challenged patents, right?

A. Yeah. I do understand that March 15th, 2007 is the priority date and what would be the date that's relevant for establishing prior art.

Q. Uh-huh. And you understand -- let me step back.

So in your three declarations in these proceedings you have some arguments regarding anticipation and some arguments regarding obviousness, right?

A. That's correct.

Q. And you have a general understanding of the anticipation and obviousness for purposes of your role in this case?

A. Yes.

Q. I think you have some like legal standards regarding anticipation and obviousness that you recite in your declarations?

A. Yes. And, again, I'm -- I'm not a patent attorney. These are elements within the

declaration that were, of course, discussed with counsel and it's my understanding through my discussions with counsel.

Q. Fair enough. So for your purposes in this case you don't have any reason to doubt that the -- the legal standards regarding anticipation and obviousness that are set forth in your three declarations are the correct ones for you to be using here, right?

A. Yeah. I feel that everything in the declaration is appropriate, and, as I said, with respect to those particular sections it's appropriate relative to my understanding through counsel.

Q. You understand that the anticipation and obviousness grounds at issue in these three proceedings are to be assessed from the viewpoint of a -- of a person of ordinary skill in the art in the field prior to that -- that date, March 15th, 2007, right?

A. I do understand that.

Q. And that's the viewpoint that you applied in this case or tried to apply, right?

A. Yes.

Q. All right.

And if I use the abbreviation POSA today to refer to the person of ordinary skill in the art, will you understand that?

A. Yes.

Q. You use that abbreviation in your declarations, right?

A. Yes.

Q. And in each of your declarations you provide a definition of the POSA in the -- in the field of the -- of the '149, '504, and '880 Patents, respectively, right?

A. Yes.

Q. And we can take a look I guess at maybe your '149 declaration as an example here. Go to paragraph 19 and you see in paragraph 19 of your '149 declaration you -- you provide your -- your definition of a POSA with respect to that patent?

A. Yes, that's correct.

Q. Now, in the second sentence here when you refer to "A POSA in the field," what's your understanding of the field for the claimed inventions of -- of the -- I guess the '149 Patent and the other two challenged patents?

A. Yeah. So I would say that the field relates to those involved with the development or

use of monoclonal antibodies.

Q. Let's look further at what you say here in paragraph 19. Maybe that's helpful. You say "A POSA in the field of the '149 Patent had knowledge of the scientific literature and skills relating to that field," the field of the '149 Patent; do you see that?

A. Yes.

Q. And you say that the knowledge and skills that a POSA would have relating to the field of this patent include the design and generation of antibodies, the complement system, and the application of antibodies as therapeutics, right?

A. Yes, I do.

MS. STERLING: Objection, form.

A. I do see that.

Q. And if we look at -- if you want to take a quick look at paragraph 20 of your '504 declaration and also paragraph 21 of your '880 declaration just to confirm that you -- you say something similar in -- in both of those declarations.

(Witness reviewing document.)

A. Yes, I see that.

Q. So let's just go back to the -- the '149 declaration, paragraph 19 as a reference point.

Why is the -- the design and generation of antibodies, why is that pertinent to the field of the three challenged patents?

MS. STERLING: Objection, form.

A. So the three challenged patents relate to the development in part or use of eculizumab or -- you know, the built-on development and use of eculizumab. So I feel that that expertise would be relevant.

Q. Why would that expertise be relevant to the development or use of eculizumab?

MS. STERLING: Objection, form.

A. So eculizumab is an antibody that was generated from a mouse hybridoma parent cell line --

THE REPORTER: I'm sorry. Mouse --

A. Hybridoma parent cell line that was then humanized through techniques that would be consistent or known to somebody who was involved with antibody development to lead to a humanized form of the particular antibody which is named 5G1.1 with a particular constant domain region where you have G2/G4 domains in the constant region of the antibody, and the expertise in antibody development I feel is relevant for understanding

the development of eculizumab.

Q. Okay. And why is the complement system that you list here in paragraph 19, why is the complement system pertinent to the field of the three challenged patents?

MS. STERLING: Objection, form.

A. So the antibody eculizumab is an antibody that binds to a complement protein C5, and so knowledge -- general knowledge of the complement system also seems to be relevant.

Q. Okay.

And what does eculizumab's binding to C5 have to do with how it works to treat patients with PNH?

MS. STERLING: Objection, form, foundation.

A. So as outlined within the declaration, PNH is a condition in which control proteins that are present in red cells are absent in patients afflicted with the condition that leads to essentially a complement attack of those red cells leading to hemolysis and release of hemoglobin. So the activity of eculizumab is to bind to C5, a critical protein in the cascade, to prevent that adverse event from occurring or to reduce the

occurrence.

Q. Uh-huh.

A. And so the knowledge of complement is relevant and it -- it relates directly to eculizumab because of its mechanism of action.

Q. So the eculizumab binding to C5 is -- is a critical aspect of its ability to treat the PNH condition?

MS. STERLING: Objection, form.

A. So it's -- it's related to its purported and then known mechanism of action. It blocks C5.

Q. Okay.

A. Or binds to C5.

THE REPORTER: I'm sorry?

THE WITNESS: Binds to C5.

BY MR. DIAMOND:

Q. And it blocks the cleavage of C5, right?

MS. STERLING: Objection, foundation.

A. So that aspect is an aspect that I'd have to admit I -- I would not say that I know perfectly well. It -- it does block the activity of C5 within the events relevant to hemolysis of red cells.

Q. Okay. And that's -- let me ask a slightly different question. A person of ordinary skill in

the art prior to March 15th, 2007 would have understood that eculizumab needs to bind C5 in order for it to work through its then understood mechanism of action to treat PNH, right?

MS. STERLING: Objection, form.

A. Yes. At the time of March 15th, 2007 it was understood that eculizumab was an anti-C5 antibody and that binding was related to its effect.

Q. It's effect in people?

A. It's a characteristic of the antibody and the antibody was known to be effective in people, yes.

Q. Okay.

Going back to what you had in paragraph 19 of your '149 declaration, why is the application of antibodies as therapeutics pertinent to the field of the -- of the three challenged patents?

A. So the patents relate to eculizumab which is an antibody that was used as a therapeutic, and for those reasons that type of expertise I think would be relevant to designation of a POSA.

Q. And if a POSA prior to March 15th, 2007 were considering the application of an antibody as a therapeutic, what sort of considerations would

they have in mind as to what would make an antibody suitable as a therapeutic?

A. It's very difficult to answer a question like that. It's really dependent on the application, and there's many, many applications even for antibodies that were developed before 2007.

Q. Fair enough. So let's -- why don't we stick with like the realm of the complement system and treating PNH or other hemolytic conditions. In -- in that context what considerations would a person of ordinary skill in the art prior to that March 15th, 2007 date had when considering the application of an antibody as a therapeutic for those purposes?

MS. STERLING: Objection, form.

A. Yeah. I would say that with respect to a POSA my opinion would be that an individual who was familiar with the use of antibodies as therapeutics, therapeutic applications of antibodies, would be an appropriate qualification, and, again, that's why I have that listed within. So it's knowledge of use of antibodies as therapeutics.

Q. Yeah. So my question was a little -- I

understand that and thank you for clarifying that. My question's a little different. My question is, again, keeping in mind -- I wanted to narrow the question for you, right?

A. Uh-huh.

Q. So now we're just talking about antibodies used as therapeutics for -- you know, directed at the complement system and -- and with the goal -- you know, for purpose of treating PNH or other hemolytic conditions like that. What considerations would a person of ordinary skill in the art as of that March 15th, 2007 date have when thinking about the application of antibodies as therapeutics for those purposes?

MS. STERLING: Objection, form.

A. Again, it's a very broad question to be able to answer. There would be many types of considerations that someone would have in thinking about the application of antibodies in this particular domain. I think that what I'm referring to here in the definition of POSA is referring to general characteristics of an individual who would be defined as a POSA with respect to, you know, the -- the case that we're referring to. So I -- it's hard for me to answer specifically what

considerations with respect to therapy someone would have for particular applications. I don't understand I guess the context.

Q. Let's step back. So the three -- the three challenged patents that we're looking at in these proceedings, they're generally directed to the use of antibodies as therapeutics, right?

MS. STERLING: Objection, form.

A. They're more specific than that, but they fall within that general area.

Q. Right. They're -- They're using specific antibodies for specific therapeutic purposes, right?

A. That's correct.

Q. All right. And so I'm not talking about a person with the benefit of those patents after March 15th, 2007. I want you to put yourself in the mindset of someone prior to March 15th, 2007. Okay?

If a person of ordinary skill in the art were thinking about developing an -- an antibody as a therapeutic for the purposes that are addressed in the three challenged patents, what are some considerations that -- that would go into -- into, you know, consideration of whether an antibody was

suitable for that purpose?

MS. STERLING: Objection.

MR. DIAMOND: And you don't have to list them all, but if any come to mind at all, that would be helpful.

MS. STERLING: Objection, form.

A. So maybe a good way to think about this would be in terms of requirements for getting approval of a drug. And so to be able to qualify to have a drug investigated in human patients within the U.S. that drug would have to have sufficient demonstration of safety within preclinical species to allow for an award of an investigational New Drug Application.

And so some of the therapeutic considerations that one would have on the path to development of a drug would relate to safety in preclinical species, and from the clinical phases of drug development you would go through a number of clinical studies leading eventually hopefully to a New Drug Application award for approval to market the drug and those clinical studies would evaluate safety and efficacy in humans to be able to justify use. And so someone interested in developing a drug would have those types of therapeutic

interests in -- you know, in considering antibody development strategies.

Q. Okay. And -- and that -- those are considerations that a person of ordinary skill in the art in the -- in the field of these patents would have had prior to -- prior to that March 15th, 2007 date, right?

A. So I don't think that's what I'm trying to say. I'm trying to say that a POSA would have experience or expertise in the use of antibodies as therapeutics. And so this is very general, and I think that that would be the level of expertise that would be appropriately associated with a POSA for these particular applications. So not, you know, very defined with respect to even approval of a drug in humans.

Q. Fair -- fair enough. I think maybe -- I'm asking a very simple question, which is the items you listed before, the considerations, the preclinical considerations and clinical considerations that you listed in your answer earlier, those are all considerations that a person of ordinary skill prior to the March 15th, 2007 date would have been aware of, right?

A. I wouldn't want it to be considered

inclusively. So I think that individuals who are interested in developing antibodies as therapeutics might, you know, in some cases have expertise in some sub-areas, but still might be appropriately considered as POSA's for the, you know, purposes of the application. I think that the best way to think of it is really just as simply as stated that I feel that these characteristics, including the application of antibodies as therapeutics, would be appropriate for a POSA and wouldn't want to restrict it more narrowly than that.

Q. Yeah. No, I'm not trying to restrict your definition of a POSA. I think we're talking past each other a little bit. So I'll try -- I'll try the question again.

Earlier you were telling me about, you know, that some -- some considerations a person of ordinary skill would have in developing a drug such as an antibody drug would be, you know, the type of information you would need in -- in preclinical studies and -- and going towards clinical trials, and I just want to understand when you -- when you gave that answer that a person of ordinary skill in the art prior to March 15th, 2007 would have been aware of those -- those general concepts you were

talking about regarding the type of information that would need to be developed to go forward into clinical trials and such, right?

MS. STERLING: Objection, form.

A. So I -- I don't, again, really want to split hairs about things. I don't think that that's really what you're interested in, but you could, for example, have a person who's involved in development of antibodies as therapeutics who was working in Europe and had no understanding of what the FDA requirements for approval of a drug would be. It might not have the same exact type of experience or background as what I stated. And so I'm thinking of this more generally that I'm talking about an individual who would have experience in the development and application of antibodies as therapeutics. So I -- I feel that broad definition is what is relevant for the definition of POSA.

Q. Uh-huh. Uh-huh.

So how -- how -- we've been dancing around this a little bit, but how do you define a person of ordinary skill in the art for purposes of these patents?

A. Yeah. So the definition I use is that a

POSA in the field of the '149 Patent would be an individual who had knowledge of the scientific literature and skills relating to --

THE REPORTER: I'm sorry.

THE WITNESS: Oh, sorry about that.

A. So "A POSA in the field of the '149 Patent had knowledge of the scientific literature and skills relating to that field including the design and generation of antibodies, the complement system, and the application of antibodies as therapeutics before March 15th, 2007."

Q. Is that it for your definition of a POSA, or is there more?

A. It goes -- it goes on. So "A POSA also had knowledge of laboratory techniques and strategies used in immunology research such as molecular cloning and protein expression and would have known of practical applications of the same. Typically a POSA would have had an M.D. and/or a Ph.D. in immunology, biochemistry, cell biology, molecular biology, pharmaceutics, or a related discipline with at least two years of experience in the field. Also a POSA may have worked as part of a multi-disciplinary team and drawn upon not only his or her own skills, but also taken advantage of

certain specialized skills of others on the team, for example, to solve a given problem. For example, a clinician and a formulation chemist may have been part of the team."

Q. And now, for purposes of assessing whether the three challenged patents were anticipated or obvious to a POSA using your definition prior to March 15th, 2007, you understand that it's wrong to rely on documents that were created after that date, right?

MS. STERLING: Objection, calls for a legal analysis or conclusion and form.

A. Yeah. So you asked a couple questions there. There are a couple parts of it. I wonder if you could maybe simplify it a little bit.

Q. Sure. So you offer opinions about whether the challenged patents are anticipated or obvious, right?

A. Yes.

Q. Okay. And you -- you understand that the relevant standard for assessing those issues is the perspective of a person of ordinary skill in the art prior to that March 15th, 2007 date, right?

A. So I'm sorry. Could you repeat that?

Q. You understand that the relevant

perspective for assessing those anticipation and obviousness issues regarding the challenged -- the three challenged patents is a person of ordinary skill in the art prior to that March 15th, 2007 date, right?

A. Yeah. So I -- the reason why I'm asking for clarification is it's hard for me to equate when you say the perspective is a person. So I'm sorry, I don't understand.

Q. Yeah. You understand that the anticipation and obviousness issues in this -- these proceedings are to be viewed from the perspective of a person of ordinary skill in the art prior to that March 15th, 2007 date?

A. I understand that, yes.

Q. All right. So you understand that, you know, documents or information that was created after that March 15th, 2007 date, that -- that's not information that was available to a person of skill in the art before that date. You're supposed to consider the -- the information before that date, right?

MS. STERLING: Objection to the extent it calls for legal analysis or conclusion and form.

A. Yeah. I do understand I think what you're

getting to. I would say that with respect to some aspects as I understand them of anticipation, for in particular the inherency issues of anticipation that you could have information that is known at a later date that reveals an inherent property of a composition that then is still relevant in an anticipation argument.

Q. Okay. And we'll -- we'll get to that, but aside from that, your general understanding is you're supposed to limit yourself to the information that was available prior to March 15th, 2007 in considering these issues, right?

A. Yeah.

MS. STERLING: Objection to the extent it calls for legal analysis or conclusion.

A. I do understand the priority date is March 15th, 2007.

MR. DIAMOND: Okay. All right. This is a good time for a break anyway. All right. Let's take a few minutes.

THE VIDEOGRAPHER: We're going off the record. The time is 10:39.

(A short break was had.)

THE VIDEOGRAPHER: We're going back on the record. The time is 10:55.

BY MR. DIAMOND:

Q. Dr. Balthasar, could you take your '149 declaration out and turn to paragraph 11.

A. Yes, I'm there.

Q. So here you see you say that -- that "Claim 1, the only claim in the '149 Patent, is directed to an anti-C5 antibody comprising a heavy chain consisting of Sequence ID 2 and a light chain consisting of Sequence ID No. 4," right?

A. Yes.

Q. And then in the next sentence you say that "The name of the anti-C5 antibody comprising Sequence ID Nos. 2 and 4 is eculizumab"; do you see that?

A. I see that, yes.

Q. And you cite two documents for supporting that sentence, AMG-1015, page 738, Boone declaration, paragraph 6, and the other document is AMG-1001; do you see that?

A. Yes, I do see that.

Q. The AMG-1001, as we saw, that's the '149 Patent, right?

A. So there's different AMG-1001's, correct?

Q. Right. For purpose -- this is the '149 declaration. So when you talk about AMG-1001 in

this declaration, we're talking about the '149 Patent.

A. Okay. Understood.

MR. DIAMOND: This is AMG-1015. It's already marked. Sure. That's fine I guess. Actually I do want that.

BY MR. DIAMOND:

Q. So you've been handed a document that is AM -- that is AMG-1015; do you see that?

A. Yes, I do see that.

Q. And if you turn to page 734 of this document using the page numbers that are in the -- on the bottom edge of the page in the center. Let me know when you're there.

A. I'm at page 734.

Q. All right. Now, you see this is the Boone declaration that you were citing in paragraph 11 of your '149 Patent declaration?

A. Yes, I see that.

Q. All right. And if you go to page 741 of AMG-1015, you see that is the signature page of the Boone declaration?

A. Yes, I do see that.

Q. And you see that the Boone declaration is dated May 11th, 2017, right?

A. Yes, I do see that.

Q. So you understand that the Boone declaration and AMG-1015 is not prior art that was available to a POSA prior to March 15th, 2007, right?

A. Yeah. I do understand that 2017 is after the priority date, but I think it's referred to in the declaration with respect to the anticipation claims and revealing inherent properties of eculizumab.

Q. So just -- we have limited time for today's deposition. So I need you to focus on my question. Okay. My question is you understand that the Boone declaration, AMG-1015, is not prior art, right?

A. So, again, I do understand that 2017 is after March 15th, 2007, but the relevance of it with respect to I think the matter relates to the inherency aspect of anticipation, and my understanding of that is that it is appropriate to refer to it for that aspect of the declaration.

Q. And who told you that?

A. It's my understanding --

MS. STERLING: Objection to the extent it gets to privileged information. You may answer

generally.

A. It's my understanding through counsel.

Q. The Boone declaration wasn't available to a person of ordinary skill in the art prior to March 15th, 2007, right?

A. The signature date is 2017, and, again, it's only being referred to within the declaration as it relates to this anticipation and inherency issue.

Q. Dr. Balthasar, could you answer my question. The Boone declaration, AMG-1015, wasn't available to a person of ordinary skill in the art prior to March 15th, 2007, yes, no, or I don't know?

A. I mean, I think I have said I do agree that 2017 is after 2007.

Q. So you won't answer my question?

A. So I think that I did. I'm not sure --

Q. I'm going to ask -- ask the question again because I want you to focus on the question I'm asking. Okay?

A. Okay.

Q. The Boone declaration in AMG-1015 wasn't available to a person of ordinary skill in the art or anyone else prior to March 15th, 2007, it didn't

exist, right?

A. Yeah. The document was signed on May 11th, 2017.

Q. Right. So as far as you know, it didn't exist prior to that date, right?

A. As far as I know -- I guess all I can say is that it was signed 11 May 2017. I don't know when it was developed.

Q. Fair enough.

If you go to page 12 of -- or paragraph 12 of your -- I'm sorry.

So if you go to page 13 of your '149 Patent declaration, you see here you have a section 6 with the header "State-of-the-art before March 15th, 2007"?

A. Yes, I do see that.

Q. And why are you summarizing the state-of-the-art before March 15th, 2007?

A. So it's included in the declaration to discuss information that was available prior to the priority date of March 15th, 2007.

Q. All right.

If you look at paragraph 45 within that section, which I think is on page 30. Let me know when you're there. Are you there?

A. Yes.

Q. You state "Before March 15th, 2007 a POSA also would have known that eculizumab contains a hybrid IgG2/IgG4 heavy chain constant region"; do you see that?

A. Yeah, I do see that. I think that there were publications available prior to this date, including the Tacken reference that gives that information.

Q. All right. My question was you see that, right?

A. Yes, I do see that.

Q. And there's two documents that you cite in support of that statement, right, AMG-1049 at pages 838 to 839 and then AMG-1034 at page 1279?

A. Yes.

MR. DIAMOND: All right. Let's take a look at that. It's the big one. Each one is like one. Yeah. All right. This is premarked. It's two volumes of AMG. I don't know if you need to mark this because this is part 2 of -- okay.

BY MR. DIAMOND:

Q. So this is -- you have two books, but you understand this is AMG-1049 that you cited in paragraph 45 of your '149 declaration?

A. Yes, I do see that.

Q. All right. Let's go to pages -- again, we're using the center page numbers on the bottom of the page. If you go to page 8 -- sorry -- 855 -- actually, go to -- go to page 830- -- 838, 839. Those are the two pages that you cite in paragraph 45?

A. Yes, that's correct.

Q. And now if you turn to page 855, you see that's the last page of the document containing those two cited pages, 838 and 849 -- 839?

A. Yes, I do see that.

Q. All right. And from page 855 of AMG-1049 you understand that the document containing those two pages, 838 and 839 that you cited in paragraph 45 of your '149 declaration, that document is dated August 2, 2011, right?

A. I see that date, yes.

Q. All right. So you understand that this document in AMG-1049 containing pages 838 and 839 that you cite, that's not prior art to the challenged patents, right?

A. Yeah. I understand that August 2nd, 2011 is a date that is after March 15th, 2007.

Q. All right. And you don't have any reason

to believe that a person of ordinary skill in the art would have had access to this document dated August 2, 2011 prior to March 15th, 2007, right?

A. That -- that's correct. I have no reason to, you know, expect that someone would have this prior to that date. I do think that the main point here is that it becomes known through an inherent property of eculizumab that it has Sequence ID No. 2 and No. 4. So I think that that is -- or has, I'm sorry, hybrid G2/G4 heavy chain.

Q. But paragraph 45 you're talking about what a person of ordinary skill in the art would know prior to March 15th, 2007, right?

A. Yes, that's correct.

Q. Fair enough. And that's why you cite AMG-1049, 838 and 839, right?

A. Yeah. 1049, 838, 839, and then also the AMG-1034, 1279 is cited as well, which is a publication from 2005, that's correct.

Q. All right.

(Whereupon a discussion was had off the record.)

MR. DIAMOND: This is premarked already.

BY MR. DIAMOND:

Q. Okay. And you've been handed 10 --

AMG-1034. This is the Tacken reference that you cite in paragraph 45?

A. Yes, that's correct.

Q. All right. We'll get back to that. Keep that in front of you for now, but first turn to paragraph 54 of your declaration, your '149 declaration, and looking at the top of page 38 in that paragraph, the sentence beginning "And as discussed"; do you see that?

A. I do see that sentence.

Q. So you say "And as discussed above, Tacken and other publications disclose that eculizumab's heavy chain has a hybrid IgG2/IgG4 constant region"; do you see that -- that sentence?

A. I do see that sentence.

Q. And you cite several references after that, the first being AMG-1033 at page 1257; do you see that?

A. I do see that.

Q. And then you cite AMG-1049, 838 to 839, which is the document we looked at earlier, right?

A. Yes.

Q. Then you cite Tacken at page 1279, right?

A. Yes.

Q. And then you cite AMG-1008 column 12,

lines 35 to 37, figure 15, right?

A. Yes.

Q. Let's take a look first at AMG-1033. So turning to the last page of AMG-1033. You can flip it all the way over. You see that the cover page of this journal article is attached?

A. Yes, I do see that.

Q. All right. And you see that AMG-1033 was published November 2007?

A. I do see that.

Q. So that's after the March 15th, 2007 priority date, right?

A. Yes. I agree that November 2007 is after March 15th, 2007.

Q. And you have no reason to believe that -- or you don't say in your declaration anywhere that AMG-1033 was available to a POSA prior to that date?

A. Yeah, I don't make any statement on that.

Q. So let's -- let's look at Tacken 1034. Do you have that in front of you?

A. Yes, I do.

Q. In the context of your involvement in these proceedings, when did you first learn of the Tacken publication?

A. I don't remember exactly. It was a publication that was discussed with counsel as we prepared the declarations.

Q. Sure. Was it something that you found or something that you got from counsel?

A. My recollection is that this probably was brought to me from counsel.

Q. And this is -- this is just a yes-or-no question here, but did you know of the Tacken publication prior to your involvement in this case?

A. It's possible that I looked at this prior to the involvement in the case, but in the context of the case I'm quite confident that it was brought to me from counsel.

Q. Okay.

Now, you understand and I think you discuss in your declarations as well that there are certain grounds of anticipation or obviousness that are being asserted by Amgen in these three proceedings?

A. I do understand that.

Q. And they're numbered like ground 1, ground 2, ground 3, right?

A. Yes, that's correct.

Q. And you understand that Tacken is not one

of the references identified as the basis for any of those grounds asserted in these three proceedings, right?

MS. STERLING: Objection, form.

A. Yes, I think that that is correct.

Q. If you go to Tacken now to the last page -- if I -- if I say Tacken to refer to AMG-1034 throughout this deposition, will you understand that I'm referring to this document AMG-1034?

A. Yes.

Q. Make things less awkward.

So if you turn to last page of Tacken, you see there's the journal cover from Blood?

A. Yes, I do see that.

Q. The journal -- the journal name is Blood, right?

A. That's correct.

Q. And this Tacken article is from the August 15th, 2005 issue of Blood?

A. That's right.

Q. If you go back to the first page of Tacken, the title of Tacken is "Effective Induction of Naive and Recall T-Cell Responses by Targeting Antigen to Human Dendritic Cells Via a Humanized

Anti-DC-SIGN Antibody," right?

A. I do see that, yes.

Q. And Tacken's abstract talks about the development of a humanized antibody that they call hD1V1G2/G4 or they call it HD1 for short, right?

A. Yeah. I see the sentence that says "Therefore we developed a humanized antibody, hD1V1G2/G4 (HD1)" and it goes on from there. I see that.

Q. And the target of that HD1 antibody is DC-SIGN, right?

A. Yes, that's correct, I see that abbreviation,

Q. DC-SIGN stands for the C-type lectin DC-specific intercellular adhesion molecule 3-grabbing nonintegrin, right?

A. Yes, I do see that.

Q. And Tacken's saying that the researcher's purpose for studying this -- this HD1 antibody targeting DC-SIGN was to explore the use of DC-SIGN as a target receptor for vaccination purposes, right?

A. It's possible. I mean, I'm sure if we look into the -- the line -- are you referring to a particular line within the abstract or article?

Q. Sure. Let's look at the abstract. If you look at the third sentence in the abstract on the left-hand side, you see Tacken says "Therefore, we developed a humanized antibody," which they abbreviate HD1, right? Do you see that?

A. Yes, I do.

Q. They say that that antibody -- that HD1 antibody is directed against what they abbreviate as DC-SIGN, right?

A. I see that, yep.

Q. And they're exploring that HD1's -- I'm sorry. They're exploring DC-SIGN's capacity to serve as a target receptor for vaccination purposes, right?

A. That's my understanding.

Q. Okay. And they're doing that by cross-linking the HD1 antibody to a model antigen which is the keyhole limpet hemocyanin or KLH, right?

A. When you say that they're doing that by, what do you mean? So that section of the abstract wouldn't refer directly to the prior evaluation of DC-SIGN as a target receptor for vaccination purposes. So I'm confused by the question.

Q. I guess what's your understanding of why Tacken was cross-linking HD1 to the KLH model

antigen?

A. For -- my understanding would be that this may be done for immunization for development of an antibody. So for development of a response, an immune response.

Q. So the goal is that the HD1 antibody is cross-linked to an antigen of interest, here the model antigen KLH, and then it binds to the DC-SIGN antibody which is associated with dendritic cells and that enables the development of a desired immune response against the antigen for vaccination purposes?

MS. STERLING: Objection, foundation, form.

A. Yeah. I don't think that's correct. So HD1 is the humanized antibody and that is linked to KLH.

Q. And what --

A. So I think the way that you stated what was being done was incorrect.

Q. How would you state what's being done?

A. Just from what they had said in the abstract. They have HD1 cross-linked to a model antigen keyhole limpet hemocyanin, and the other elements of the paper is not, you know, an area

that I've given much attention to.

I have to admit my main interest in Tacken relates really to what we referenced within the declaration, which is that it identifies clearly that eculizumab is an antibody that has an IgG2/G4 constant region.

Q. All right.

A. So I -- I have to admit I haven't looked at the fine details to be able to describe clearly and completely all aspects of what they've done. My interest was mostly relating to the structure of eculizumab.

Q. That's fair enough. So when you -- when you read Tacken in the context of this case, you focused on the discussion in Tacken of eculizumab, right?

A. That's correct.

Q. And you focused on Tacken's discussion of eculizumab because you knew that the challenged patents at issue in this case were -- were directed to eculizumab, right?

MS. STERLING: Objection, form, misstates witness's testimony.

A. Yeah. I was trying to think of what a POSA would do to try to develop eculizumab. And so

a typical thing that I would do if I was -- if I had that interest would be to search literature for information about eculizumab, and this is a paper that would come through that type of search and the paper gives information that is consistent with the statement in the declaration that eculizumab is known to be an antibody with an IgG2/IgG4 constant region.

Again, the reason why this is important within the declaration is relating to the structure of eculizumab, and the knowledge that eculizumab has this structure is something that was indicated elsewhere, including statements made by Alexion stating that at 2002 this was something that was well appreciated by people in the field. Again, the interest that I have in this paper all stems to the relationship of eculizumab structure.

Q. Right. And you're considering eculizumab structure in the context of this case because the patents at issue in this case relate to eculizumab?

MS. STERLING: Objection, form.

A. I'm trying to think again of what a POSA would be doing to be able to attempt to generate eculizumab, an effective antibody for treatment of PNH, and thinking of how somebody would go about

trying to collect information about eculizumab and move forward, and that's the relevance of this paper is that it gives information about the structure of eculizumab.

Q. As you said before, you didn't obtain this paper from a search you did yourself. You probably got it from counsel, right?

A. Yeah, that's my recollection. I may have seen this in the past. I have to admit that my group had had some interest in complement for projects that we were undertaking in the early 2000s. It's possible that I came across this paper for other reasons, but in the context of this particular case I'm quite confident that it came from counsel.

Q. You didn't mention anything in your declaration about any personal involvement you had with this paper, right?

A. I didn't mention it. Yeah, I don't have -- I'm not an author and, you know, it's not anything that we contributed to if that's what you mean by "personal involvement."

Q. There's no mention of eculizumab in the title or abstract of Tacken, right?

A. Yeah, that's correct. I -- I don't see

mention of eculizumab in the title or within the abstract, but this is -- within the abstract there is reference to G2/G4 which I think would be of interest to people interested in eculizumab.

Q. Right. For purposes of this deposition I really need you to focus on my questions.

A. Sure.

Q. Your counsel's actually able to ask you questions later if they have anything that they want to follow up on. So I really need you to focus on my question.

There's no mention of C5 in the title of Tacken, right?

A. Yeah, I don't see any mention of C5 in the title of this paper.

Q. There's no mention of C5 in the abstract of this paper, right?

A. Yeah. I've read through the abstract quickly and, to the best that I can tell, I do not see any reference to C5 within the abstract of the paper.

Q. You don't see any reference to paroxysmal nocturnal hemoglobinuria or PNH in the abstract or title of Tacken, right?

A. No, I don't see that reference eculizumab

or to PNH or to C5 in the title or the abstract, but it is, again, referenced within the article. C5, eculizumab are both referenced within the article.

Q. Sitting here right now, you're not aware of PNH being referenced in the article, are you?

A. Yeah. Again, I see reference to eculizumab, IgG2, IgG4, C5, and quickly looking through --

Q. I'm asking about PNH, not --

MS. STERLING: The witness was still answering the question. Could you let him finish.

MR. DIAMOND: The witness is not answering my question.

A. But quickly looking through -- let me see.

Q. I am not going to trouble you to read every word of it right now, you know, because we have a bunch of things to talk about, but sitting here right now, you're not aware of any instance in which PNH is mentioned in Tacken, right?

A. I would say it's correct. I did review this article in preparing my declaration and in preparing for the deposition, but I don't remember every word. And so I can't say with confidence whether or not PNH is referred to or not.

Q. Okay. Sitting here right now, you don't recall?

A. Yes. Sitting here right now, I don't have any confidence in saying whether or not it's present within the article.

Q. Okay. And you didn't mention anything about Tacken talking about PNH in your declarations as far as you recall, right?

A. Yeah. As far as I recall, I did not. It was really just referred to as referring to the sequence or composition really of eculizumab as being, you know, this IgG2/IgG4 version of 5G1.1.

Q. Let's look at what you're actually relying on in Tacken on page 1279.

A. Sure.

Q. So what you're relying on in Tacken for your opinion that Tacken described eculizumab as containing a hybrid IgG2/IgG4 constant region, that is the last sentence under the paragraph with the heading "Recombinant antibodies" in the left-hand column of this page?

A. Yeah, I do see that sentence. It says "An isotype control antibody, h5G1.1-mAb" and then this "(5G1.1, eculizumab, Alexion Pharmaceuticals) containing the same IgG2/IgG4 constant region is

specific for the human terminal complement protein C5."

Q. Uh-huh. So that's the sentence that you're focused on in Tacken, right?

A. Yeah. This sentence from the paper does give the name eculizumab, it relates this to the structure of the IgG2/IgG4 constant region, and that's what we're talking about within the declaration.

Q. Okay. That sentence in Tacken misspells eculizumab, right?

A. That's correct.

Q. A POSA would have seen -- I guess would a POSA have known the correct spelling of eculizumab?

A. I think a POSA would have recognized that as eculizumab partially because the reference to 5G1.1, Alexion Pharmaceuticals being referenced, the fact that it's a humanized version of that antibody, I think all of those things point to eculizumab, and I don't believe that a POSA would have any doubt that this is just a simple misspelling of eculizumab.

Q. Okay. But a POSA would have recognized that as a spelling error?

A. Yeah. I think a POSA would have

recognized this as referring to eculizumab.

Q. And so they would recognize it as a spelling error?

A. So yeah. Again, I think that the reference here is clear that what this is referring to and people would accept this as eculizumab, yep.

Q. That sentence that you just read is the only mention of eculizumab in this Tacken paper, right? The only time the word eculizumab appears in this paper?

A. I think that that is possible, but I wouldn't want to, again, respond with any type of confidence without reading through every single word. I don't remember every word.

Q. Fair enough. Sitting here right now, that sentence -- that one mention of eculizumab in that sentence is the only mention of eculizumab that you're aware of in Tacken, right?

A. I guess I would say sitting here right now I don't remember every word word for word. I know that this sentence directly lists eculizumab. It indicates that it's the antibody humanized 5G1.1, gives information about the constant region structure of being G2/G4, indicates that it's specific for the human terminal complement protein

C5. I know that for sure, but I can't really say with confidence about everything else in the paper.

Q. Sitting here right now, that's the only mention of eculizumab that you're aware of, right?

A. Yeah. Sitting here right now, I see this directly in front, I know this is what I focused on in the declaration, this is why we reference to it, and I see this definitely here as being a reference to eculizumab.

Q. And there's a reference No. 19 cited in support of this sentence that you're relying on?

A. That's correct.

Q. All right. And reference 19 is the Thomas paper from 1996, right?

A. Yes, that's correct.

Q. And I guess just for the record, you see that there's the reference 19 to Thomas appears on page 1285 of Tacken? That's where the full citation to Thomas appears?

A. Oh, I'm sorry. Yes. I do see Thomas listed on page 1285, yes.

Q. So you've been handed Amgen -- Exhibit AMG-1023, and you recognize this as the Thomas paper we were just discussing?

A. Yes, I do see that.

Q. This is the Thomas paper that's cited as reference 19 in Tacken, right?

A. Yes, that's correct.

Q. Thomas is the reference you understand that describes the development of, among other things, a humanized IgG4 isotype antibody based on the 5G1.1 mouse antibody, right?

A. Yeah. This is a paper that does talk about humanization of anti-C5 antibodies in single-chain Fv's and, as you say, there's a number of constructs that are created within the paper, and I'd have to say that I view this as an important paper talking about humanization of this type of antibody. It's an important methods paper.

Q. You agree that Thomas disclosed generating an intact humanized h5G1.1 antibody by transferring the CDR regions of murine 5G1.1 into a human antibody sequence, right?

MS. STERLING: Objection, foundation.

A. Yes, I understand.

MR. DIAMOND: Counsel, first of all, you have to stop these coaching objections. Second of all, I'm reading from his declaration. So I don't understand what the foundation objection is.

MS. STERLING: Well, first, I'm not giving

coaching objections. I'm using one-word objections like the Board has instructed us to. I wasn't aware you were pointing to his declaration. You hadn't mentioned that.

MR. DIAMOND: Well, then don't --

MS. STERLING: I was looking at the document you were directing him to.

MR. DIAMOND: Then don't -- don't object.

THE REPORTER: One at a time.

MR. DIAMOND: He's changing his answer when you object. Don't object.

MS. STERLING: My intention is not to coach the witness. My intention is to keep a clean record.

MR. DIAMOND: We'll see.

MS. STERLING: Thank you.

BY MR. DIAMOND:

Q. Let's look at paragraph 47 of your declaration.

A. So this is the declaration '504 or '149?

Q. '149.

A. Okay.

Q. Looking at your second sentence in the declaration, you say "For example, Thomas, et al.'s

publication in the Journal Molecular Immunology in 1996, AMG-1023, disclosed generating 'an intact humanized 5G1.1 antibody'"; do you see that?

A. I do see that sentence in the declaration.

Q. And you cite to page 1396 of Thomas for that? I guess 13- -- pages 1389 and 1396, right?

A. Yes, I see that citation within the declaration.

Q. All right. And you understand that the humanized antibody described in Thomas, the intact humanized antibody described in Thomas is an IgG4 isotype antibody, right?

A. So, again, I know that there's a number of constructs that they developed within the paper including SscFv's. Offhand I can't say whether or not there's more than one intact structure that's present. I know that there are a number of constructs here, but I also, you know, see the sentence from the declaration that you're referring to.

Q. Yeah. Let's -- let's make it really easy. Looking at page 1396 of Thomas -- are you there? Let me know when you're there.

A. Yes.

Q. There's a header "Construction of a

Humanized h5G1.1 Antibody," right?

A. I do see that header, "Construction of a humanized h5G1.1 antibody."

Q. Thomas says "Having demonstrated the effective humanization of the 5G1.1 variable regions, an intact humanized antibody (IgG4 isotype) was constructed and produced in 293 EBNA cells"; do you see that?

A. I do see that sentence.

Q. That's the only humanized antibody that -- the only intact humanized antibody that Thomas describes, right?

A. You know I, again, have looked at this article in preparing for the declaration. I, you know, could see that it's possible that there's only one intact antibody described, but I wouldn't want to respond to that with confidence without reading through everything.

I have to say that my main focus on this paper was its significance with respect to the method of humanization, CDR grafting, which was really being developed around this time. So this is one of the early examples of developing a humanized antibody by CDR grafting approaches. So that's really the significance of this paper in my

mind.

Q. If you go to page 1399 of Thomas, AMG-1023 -- for purposes of today if I say Thomas, you understand I'm referring to this AMG-1023?

A. I do, yeah.

Q. Look in the right-hand column. Do you see several lines down a sentence beginning "As the half-life of the h5G1.1 scFv." Do you see that?

A. So are you within the --

Q. I'm trying to point you to the beginning of the sentence so to make sure you're looking at the same place as me.

A. I know. I'm trying to find it as well. So --

Q. It's about a third of the way down in the right-hand column, it says "1996" and then it says "As the half-life of the h5G1.1."

A. I do see that now, yes.

Q. Great. Now that you're there with me, Thomas says here on page 1399 "As the half-life of the h5G1.1 scFv is likely to be too short to allow for chronic use in humans, a full-length humanized antibody was also constructed," right?

A. I do see that, yeah.

Q. They don't say that multiple full-length

humanized antibodies were constructed, right?

A. I just see the sentence as you read it, that "As the half-life of the h5G1.1 scFv is likely to be too short to allow for chronic use in humans, a full-length humanized antibody was also constructed."

Q. All right. And they say the human IgG4 isotype was chosen, right?

A. I do see that.

Q. That's what a POSA would understand reading this paper, right?

A. I think that a POSA would be able to read that sentence, yes.

Q. And they would understand that it means that Thomas and the researchers in Thomas chose a human IgG4 isotype for the full-length humanized antibody constructed in this paper, right?

A. Well, you know, just to give an analogy, I think what you're saying is that if you take the first step that that's the only step that would be taken, and what I'm saying is that I know what it says, that they developed a humanized IgG4 version. And I'm just saying that I don't have confidence that there was not other things done, that other steps weren't taken, and I would have to feel

confident looking at the entire thing word by word to really understand that. I do see all the things that you've mentioned.

Q. Yeah. I mean, sitting here today, you can't point to any other intact humanized h5G1.1 antibody described in Thomas aside from the IgG4 isotype antibody, right?

A. I would be happy to look through it. So...

Q. Without sitting here and rereading every word of Thomas, you can't point to any other humanized -- intact humanized antibody described in Thomas aside from the IgG4 isotype antibody, right?

A. Again, I have to apologize in that I don't know this paper to the point of being able to say, you know, a hundred percent sure that it doesn't exist. So, for example, in figure 10 they're talking about what appears to be intact antibodies, there's mouse -- or m5G1.1-mAb, there's a designation for h5G1.1 HuG4 mAb, there's another version that is ch3F4 HuG4 mAb. These appear to be intact mAbs, and I'd want to go through the paper very carefully to be sure about whether or not this is correct, that there's only one intact humanized version of the mAb that they're talking about.

I see reference to many things within the paper that refer to the designation of mAb indicating an intact mAb, and I don't know offhand if -- you know, to what extent they're referring to humanized versions.

Q. You understand you're here today to provide testimony regarding your declarations and the references that you cite in your declarations in this case, sir?

A. I completely understand that, and as I've mentioned before, I have to admit that my interest in Tacken is that it's a leading paper that talks about the methods of humanization. So CDR grafting in the context of the C5 antibody, which was really new at that time and that's why it's important and was referred to so often in the documents that are relevant to this particular case, it's really a methods paper talking about humanization.

Q. When you said -- I think you just said your interest in Tacken in the answer, but I think you misspoke. You meant your interest in Thomas?

A. It is the interest in Thomas that I was referring to.

Q. I'll give you one more chance here. If you can point me to anything let me know, but

sitting here right now with all the time that you spent preparing for your deposition today and all the time that you spent reviewing the references and preparing your declarations, you can't point me to any other intact humanized 5G1.1 antibody described in Thomas aside from the IgG4 isotype antibody, right?

A. I'm sorry. Could you repeat the question.

MR. DIAMOND: Could you please read back the question.

(Record read as requested.)

A. Yeah. So sitting here right now, I have to admit I don't have complete recollection of every word within the Thomas reference to be able to say whether or not there are other intact humanized 5G1.1 antibodies referenced, that's correct.

Q. And if Thomas did identify -- well, let me ask you this question. You can't point me to any place in Thomas where they identify a humanized 5G1.1 antibody in Thomas with a hybrid IgG2/G4 constant region, right?

A. So, again, I have to admit that my interest in Thomas is really as it relates to

methods of humanization, the CDR grafting approach that was used in development of eculizumab, and I really have, you know, focused on the paper for that purpose, and I can't say with confidence, you know, without going through word by word -- I'd be happy to do it if you would want -- whether or not there's any reference to an antibody with a hybrid G2/G4 domain that's present.

Q. If Thomas did talk about a humanized 5G1.1 isotype -- if Thomas did talk about a humanized 5G1.1 antibody with an IgG2/G4 isotype, that would have been of interest to you in drafting your declarations, right?

MS. STERLING: Objection, form.

A. Yeah. It's hard to kind of think about things in hypothetical terms, but I would have to say generally I would be of interest in that and that's really because eculizumab would be -- is known to be an antibody with a hybrid G2/G4 domain. That was established within the field, it's known by Tacken, and even Alexion's own individuals refer to this in several places.

So there is within the declaration one sentence that I think is particularly relevant with respect to this. It's -- if you look at --

Q. Is this a sentence regarding the antibody that Thomas discloses?

A. It's referring to what is known in the state-of-the-art with respect to eculizumab.

Q. Yes. That's not my question.

A. Okay.

Q. My question was about Thomas.

A. Yeah. I thought that you were asking about my interest in G2/G4 antibodies.

Q. I was asking about your interest in -- if Thomas disclosed -- let me ask you a different question.

Sitting here right now, you're not aware of any place in your declaration that you offer any opinion that Thomas disclosed any intact humanized antibody other than the IgG4 antibody, right?

A. When you say "humanized antibody," do you mean intact antibody?

Q. Sitting here today, right now where you are, you're not aware of anyplace in any of your three declarations where you offered any opinion that Thomas discloses any intact full-length humanized 5G1.1 antibody other than the IgG4 isotype antibody that we've seen?

A. To the best of my recollection, within the

declaration I don't make reference to Thomas as giving information about additional intact antibodies aside from those mentioned I guess directly within the declaration. So you're asking me if I have in the declaration definition beyond something. Beyond what? So if you could refresh my memory about the question.

Q. Go to paragraph 47 of your '149 declaration and one, two, three, four lines up from the bottom you see a sentence beginning "Thomas states"?

A. Yes, I do see that.

Q. You say "Thomas states that the humanized antibody was an 'IgG4 isotype' and refers to it as 'h5G1.1 HuG4,'" right?

A. Yes, I do see that.

Q. Do you stand by that opinion, sir?

A. I completely stand by the statement as written in the declaration that "Thomas states that the humanized antibody was an 'IgG4 isotype'" --

THE REPORTER: Slow down, please.

A. -- "and refers to it as 'h5G1.1 HuG4.'"

Q. If you go back to Thomas on figure 10 on page 1399.

A. Yes, I'm there.

Q. You see there's circles indicating h5G1.1 HuG4 mAb?

A. Yes, I do see that.

Q. And you understand that that refers to the IgG4 isotype intact humanized antibody that Thomas developed, right?

A. Yes, I do see that it's designated as the h5G1.1 HuG4, which is referring to this humanized IgG4 isotype antibody, yes.

Q. Now, Tacken when it talked about using an antibody h5G1.1 mAb, it was using that as an isotype control antibody, right? Maybe I can just direct you to it if you can't answer off the top of your head.

If you look back at the sentence we were focusing on in Tacken at page 1279, lower left-hand corner, you see it refers -- that sentence refers to h5G1.1-mAb as "an isotype control antibody"; do you see that?

A. I see the sentence that you're referring to. It reads "An isotype control antibody, h5G1.1-mAb (5G1.1, eculizumab, Alexion Pharmaceuticals) containing the same IgG2/IgG4 constant region is specific for the human terminal complement protein C5," yes.

Q. So yes, that sentence is referring to h5G1.1-mAb in the context of this study as an isotype control antibody, right?

A. Yes. Eculizumab is, I agree, referred here because it's known and has an IgG2/4 constant region and so is used as an isotype control antibody for the study.

Q. Tacken wasn't studying h5G1.1-mAb to determine whether it binds C5 or treats PNH, right?

MS. STERLING: Objection, form.

A. Yeah. I think that this was well established in the art. So they refer to eculizumab as an antibody that has the structure, the G2/G4 constant region, and that it binds to the human terminal complement protein C5.

Q. So Tacken doesn't cite to any data showing that any h5G1.1 antibody with a hybrid G2/G4 isotype has C5 binding activity, right?

A. So are you asking if 5G1.1 is necessarily referring to a G2/G4; is that what you're asking?

Q. I'm just asking if there is C5 binding data regarding that isotype control antibody in Tacken? Did Tacken report the results of any C5 binding study?

A. Yeah. There, to my knowledge, is no such

data, and the reason is that eculizumab is only used here, you know, as something that's not developed but is being used as a reference compound and that eculizumab is well known and they talk about some of the well-known characteristics of eculizumab including that it does bind human complement protein C5 and that it has the structure that they're interested in in this paper, which is the G2/G4 structure and it's, you know, just an indication of an antibody that has these known characteristics.

Q. If you go back to paragraph 54 -- go to paragraph 54 of your declaration, your '149 declaration, and we looked at this before. I'm bringing your attention again to the sentence at the top of page 38 where you say "And as discussed above, Tacken and other publications disclose that eculizumab's heavy chain has a hybrid IgG2/IgG4 constant region." You see that again, right?

A. Yes, I do see that sentence.

Q. And we've looked at AMG-1033, we've looked at AMG-1049 that you cite, we looked at Tacken AMG-1034. The other reference that you cite here -- or I guess the last reference that you cite here in support of this sentence is AMG-1008,

right?

A. Yes, that's correct.

MR. DIAMOND: All right. This is premarked 1008.

BY MR. DIAMOND:

Q. So you have AMG-1008 in front of you, sir?

A. Yes, I do.

Q. This is the Mueller publication that you rely on as the basis for some of your obviousness grounds in these proceedings?

A. Yes, that's correct.

Q. And let's take a look at what it is that you are citing to in paragraph 54 of your '149 declaration in Mueller. You cite to page 12 first. Let's take a look at that. And on page 12 you cite to lines 35 to 37. Do you see that sentence in Mueller describes two antibodies with the prefix h5G1.1, right?

A. Yes, I do see that.

Q. One of those is h5G1.1 with the HuG4 isotype and the other is h5G1.1 with the HuG2/G4 isotype, right?

A. That's correct.

Q. And Mueller was using both of these h5G1.1

antibodies as control antibodies, right?

A. So I know that both are referred to within figure 15. So I know both are present within the paper -- or within the document.

Q. In other words, this isn't the paper -- Mueller isn't the paper that's studying C5 binding of antibodies. It's studying binding of antibodies to VCAM, right?

A. Yeah. Again, so the knowledge that I have in Mueller is mostly focused on the paper as a source of information for sequence for eculizumab. That's really what I focused on. So it does give information on eculizumab sequence, including this G2/G4 constant domain structure. That's really where I focused on. So to be able to answer questions outside of that, I have to admit I'm not completely conversant with all aspects of the document.

Q. Fair enough.

How did you get Mueller for purposes of this case? Was it something you found or something that was given to you by counsel?

A. This was something that was brought to me by counsel.

Q. Okay. And you understand you were

provided Mueller because it was a reference that relates to eculizumab?

MS. STERLING: Objection, calls for speculation.

A. Yeah. So I think, again, in thinking about this and its relevance in the context of a POSA at the time of, you know, interest, which is in 2007, I think that a POSA would be interested in looking at names or synonyms of eculizumab and eculizumab itself to be able to get information about eculizumab.

One of the designations that was used for eculizumab is 5G1.1 or h5G1.1 which appear within the Mueller reference, and that's why I think I would be directed to it as a POSA interested in eculizumab.

Q. Let's talk about that. So h -- h5G1.1 -- looking at Mueller at the bottom of page 12, h5G1.1 with the HuG4 isotype, that's what we saw in Thomas, right?

A. Yeah. There was reference to that in Thomas, correct.

Q. Okay.

A. Could I maybe talk a little bit about this naming of 5G1.1 and what it means?

Q. That's not my question right now. I may ask you about that later.

A. Okay.

Q. We can get to that, but right now just stick with my questions.

A. Sure.

Q. You understand that the IgG4 isotype of the antibody that we discussed that was described in Thomas, the intact antibody body of Thomas, and the h5G1.1 HuG4 that maybe that's described here in Mueller, that's a different antibody with a different sequence than the antibody that's recited in the claims of the challenged patents at issue here, right?

MS. STERLING: Objection, form.

A. Yeah. I do understand that eculizumab is an antibody with a human G2/G4 constant domain and that antibodies that are -- humanized antibodies with a G4 antibody -- I'm sorry -- constant domain are necessarily not equivalent and would be different.

Q. Okay.

So now, Mueller uses the term h5G1.1 to refer to at least two different antibodies in this sentence we're looking at on the bottom of page 12,

right?

A. It's true. Again, the 5G1.1 name is something that derives from the strategy that is typically used in antibody development. So --

Q. The humanized 5G1.1?

A. So this antibody was generated initially through development of a mouse antibody, and at that time in the late 1990s that process typically proceeded with immunization of mice, development of hybridomas, and then pleading out those hybridomas in microplates. The microplates are often numbered because there would be many of them.

The microplates have rows which are labeled for 96 well plates A through H and have columns 1 through 12. So 5G1 typically would be referring to plate 5, row G, column 1. So that name relates to the cells that were within that particular well of the plate.

So it's very typical through antibody development to be able to use that name to refer to the parent hybridoma, the antibody that relates to that, and to use variants of that name as you develop variants of that antibody. So h5G1.1 is referring to variants of that particular antibody.

And as you go forward in antibody

development eventually you select a candidate that would be a lead candidate maybe for clinical development, and at that point you would assign a name to that, you would request a name. That's eculizumab.

So there's many G5.1's, but as you get to eculizumab, it refers to a specific 5G1.1 that has a particular sequence and structure and that is known to be the G2/G4 sequence and structure which is given in Tacken and is referred to directly by Alexion as saying that it is well known by people of ordinary skill in the art that by 2002 it's accepted that eculizumab had this particular structure.

So when you're asking about does h5G1.1, is this a humanized antibody that has a G4 in this particular context, that's true, but it would be known by people at the time of interest, March 15, 2007, that eculizumab is a specific humanized 5G1.1 that has the G2/G4 sequence.

Q. So let me just break that down. You just said a lot. I do need you to make your answers shorter because it just -- so we could, you know, address each point.

So when a -- when a -- so eculizumab,

that's a nonproprietary name? I guess let me -- let me take that back.

When an antibody is given a unique name like eculizumab, you're saying that that then refers to one specific antibody with one specific primary amino acid sequence?

A. That is the case that eculizumab would be referring to a specific version of the 5G1.1 antibodies and particularly humanized 5G1.1 antibodies, and in this case eculizumab is known to be the version that is humanized with a G2/G4 constant domains.

Q. And going back and to start at the beginning of that process that you described --

A. Yeah.

Q. -- so initially there was a 5G1.1 hybridoma?

A. That's right.

Q. Which was developed from the fusion of -- of two cells that then produced that antibody?

A. That's correct.

Q. And the resulting antibody obtained from that, that's the 5G1.1 antibody, right?

A. Yeah. So it, again, really refers to typically the plate, the row, the column that the

hybridoma exists within, and then that same label that relates to that plate, row, column is used for the hybridoma is often used to describe the antibody that comes from that hybridoma and often that same name is used within description and often as the description of many different variants of that initial antibody. That's very common in the antibody development field.

Q. So there's an 8 -- sorry. There's a 5G1.1 hybridoma named because of its location on the plate which produces a 5G1.1 antibody, and then as development proceeds if humanized versions of that -- that mouse 5G1.1 antibody are developed, then they are designated h5G1.1 to indicate the humanization?

A. Often, but not always.

Q. In this case I guess.

A. So there's many references to eculizumab as 5G1.1. So in this particular context the H is referring to humanized within this document, but there's other indications that you would see where just 5G1.1 would be used. And so the symbol or name that is used there isn't really what helps to identify. It's eculizumab, at that point it's tied to a particular structure which is, you know, the

humanized G2/G4 construct of the 5G1.1 antibody.

So it is true that you'll see different designations for 5G1.1, but in the context of eculizumab, like in the Tacken reference, it's referring to the specific construct, the G2/G4 construct.

Q. Let's put Tacken aside for a minute. We'll get back to Tacken, but put Tacken aside for a second and just focusing here on Mueller and then the sentence we're looking at in Mueller. Mueller here doesn't tell a POSA which one of these h5G1.1 antibodies, if any of them for that matter, is eculizumab by that name, right?

A. That's correct. Mueller is a document that comes from I believe 1997 which predates the designation of eculizumab. So the description is eculizumab and its relationship to structure is only known later in time, but the appropriate time, again, is 2007. And if you look even at Alexion's statements, they say that well before that, even in 2002 it was well known to anyone of ordinary skill in the art at the time of filing priority applications in 2002 that eculizumab has this G2/G4 FC portion.

Q. What document are you relying on for that

statement about 2002?

A. This is referenced within the declarations at different places. I happen to be looking at the declaration for '504 in --

Q. That's fine.

A. -- paragraph 46.

Q. Paragraph 46 of your '504 declaration?

A. Yeah.

Q. Let's take a look.

A. It relates to U.S. Patent Application No. 11/127,438.

Q. All right. That's Amgen -- that's AMG-1049 that we were looking at earlier?

A. That's correct.

Q. And that was the document we saw that was dated in 2011, right?

A. That's right.

Q. All right.

A. And it's referring to the state-of-the-art in 2002. That's the statement of interest.

Q. But the statement was made in 2011, right?

A. Yeah. What the statement is, again, is that "It was well known to one of ordinary skill in the art at the time of filing priority applications in 2002 that eculizumab has a G2/G4 FC portion" and

it goes on from there, but that's -- what's relevant is that Alexion says that this is well known to anyone of ordinary skill in the art that eculizumab is this particular version of the 5G1.1 antibody that has the G2/G4 construct.

Q. But Tacken is the only document that you cite dated prior to March 15th, 2007 that says eculizumab -- that word and associates it with IgG2/G4, right?

A. So it's true that Tacken does include eculizumab and does include that sequence information. There's structure information about the humanized G2/G4 structure.

Q. And you don't cite any other documents dated prior to March 15th, 2007, sir, that associate the word eculizumab with IgG2/G4 structure, right?

A. That's right. There's no documents that I cite in addition to Tacken that made that designation, but it is also correct that Alexion indicates that it's well known to anyone of ordinary skill in the art that by 2002 that it was well known that eculizumab has this G2/G4 FC structure.

Q. And do you know what document, if any,

supports that statement regarding eculizumab being associated with IgG2/G4 being known as of 2002, aside from Tacken? Can you point to any other document?

A. I haven't done an exhaustive review of the literature to be able to look at every possible paper, but -- you know, so what I was focused on is what a person of ordinary skill in the art would know at the time of interest, which is March 15th, 2007. By that time there were references, like the Tacken reference, that indicates that eculizumab has the G2/G4 sequence. Alexion makes a statement by 2002 that this was well known to anyone of ordinary skill in the art, and I accept that as being demonstration of knowledge in the field at that time.

Q. Let's do this. Now, you agree that Mueller doesn't study the C5 binding of the h5G1.1 controls, right?

A. Again, without looking through it in great detail, going word for word, I wouldn't be able to say with absolute certainty, but I will say that that's my understanding, that Mueller does not study C5 binding.

Q. And same thing with respect to Tacken

without going through word by word, but you understand that Tacken didn't study the C5 binding of the h5G1.1 control, right?

A. Again, Tacken -- I have to admit that my main interest in Tacken is as we talked about before, is that it's a key reference that does tie eculizumab to structure saying that eculizumab is this G2/G4 constant domain antibody. That's my focus on Tacken.

Q. So, sir, I'm not asking you about -- you've got to focus on my question. Okay? Your focus today is my question, okay, and my question is that without going word by word through Tacken, you understand that Tacken didn't study the C5 binding of the h5G1.1 control, right?

A. I apologize for saying the same thing, but I think we did say the same thing about this before, is that without going word for word all the way through I wouldn't want to make any statement of confidence with respect to what is or was not considered within Tacken. And the reason for that is that my interest in Tacken --

Q. Sir, we don't need to get into your -- you need to answer my question. Is the answer I don't know? If the answer is I don't know, say I don't

know.

A. So I --

Q. Sitting here today I don't know, is that the answer?

A. So I would not be able to answer with confidence.

Q. Sitting here today you don't know?

A. So sitting here today I would not feel comfortable answering that question with confidence without reading through line by line, word by word.

Q. Sir, you have to give me an answer. So yes is an answer, no is an answer, I don't know is an answer. I don't have confidence, that's not an answer.

So sitting here today, can you answer my question, yes, no, I don't know?

A. Yes. My prior answer that I wouldn't have complete confidence in answering your question without reading through word by word, line by line is the correct answer.

Q. And therefore you don't know the answer to my question sitting here today, right?

A. Yeah. So therefore I would not feel confident making any answer to your question without reading through line by line, word by word.

Q. Respectfully, sir, it's not your choice whether or not to answer my questions. You're here under oath today and it's your obligation to answer my questions. You can't decide not to answer my questions because you don't feel confident. If the answer is I don't know, you can say that, but you have to give me an answer.

A. Okay.

Q. So with that understanding -- I'll go back to my question again. Sitting here today, as far as you're aware of what Tacken describes, you're not aware of any study of C5 binding of the h5G1.1 control in Tacken, right?

A. So I don't know that I recall that as being your question, but to answer that question if that's what you would like me to do --

Q. My question's really simple. It's sitting here today with Tacken, as far as you understand Tacken and what's in it, you understand that Tacken doesn't study the C5 binding of the h5G1.1 control, right?

A. And I think that you wanted me to say either yes or no, and I guess --

Q. Or I don't know if you don't know.

A. My answer is yes, I agree that I do not

know the reference word for word.

Q. And therefore you don't know the answer to my question?

A. Yes, because I don't know the reference word by word all the way through, I wouldn't feel confident giving you an answer to the question.

Q. If you don't feel comfortable saying yes or no, then your answer is I don't know. Can you say I don't know? It's okay.

A. I don't know.

MR. DIAMOND: Yeah, sure. Let's take a break.

THE VIDEOGRAPHER: We're going off the record. The time is 12:14.

(A short break was had.)

THE VIDEOGRAPHER: Going back or the record. The time is 12:31.

BY MR. DIAMOND:

Q. Dr. Balthasar, could you turn your '149 Patent declaration to paragraph 12. Are you there?

A. Not yet. Yeah.

Q. All right. You say based on your review of the prior art, "Eculizumab was a well-known anti-C5 antibody that multiple clinical trials had

demonstrated to be effective in treating PNH long before March 15, 2007," right?

A. Yes, I see that sentence.

Q. And you go on to discuss three references describing the safety and efficacy of eculizumab in treating PNH prior to March 15th, 2007, the Bell reference, AMG-1005, the Hillmen reference in the New England Journal of Medicine, AMG-1004, and the Hill '05 reference, AMG-1047, right?

A. Yes.

MS. STERLING: Objection, form.

BY MR. DIAMOND:

Q. Okay. If you agree that the New England Journal of Medicine is a prestigious publication in the medical field, right?

A. Yes.

Q. And as you state in paragraph 12, the Bell, Hillmen, and Hill references all disclose that "Eculizumab was 'well tolerated' and a 'particularly useful' therapeutic for treating PNH," right?

A. Yeah. I do see that -- that sentence at the end of that paragraph.

Q. Okay.

MR. DIAMOND: Let's mark these. So we've

got 1005, 1004, and 1047.

MS. STERLING: Excuse me, Counsel. While you're finding that document, can we go off the record for a second? The live feed has stopped.

MR. DIAMOND: Sure.

MS. STERLING: Is that okay?

MR. DIAMOND: Yeah.

MS. STERLING: Just while you're organizing your document.

MR. DIAMOND: That's fine. Yeah.

MS. STERLING: Can we go off the record?

THE VIDEOGRAPHER: Yeah. We're going off the record. The time is 12:33.

(A short break was had.)

THE VIDEOGRAPHER: Going back on the record. The time is 12:34.

BY MR. DIAMOND:

Q. So Dr. Balthasar, you have in front of you AMG-1004, AMG-1005, and AMG-1047, right?

A. Yes, that's correct.

Q. And these are the Hillmen 2004, Hill 2005, and Bell references that you discuss in your declarations in this case?

A. Yes, that's correct.

Q. In paragraph 35 of your '149 declaration,

can you turn there and let me know when you're there.

A. I'm sorry. The paragraph number?

Q. 35.

A. Yes, I'm at that paragraph.

Q. You're discussing the Hillmen 2004 paper in this paragraph, right?

A. Yes, that's correct.

Q. And, as you state here, "Hillmen stated that its goal was to test the clinical efficacy of eculizumab, a humanized antibody that inhibits the activation of terminal complement components in patients of PNH," right?

A. Yeah. I see that direct quote from the declaration.

Q. That's -- those are your words, right?

A. That's right.

Q. "Hillmen's trial enrolled 11 patients and each patient received infusions of eculizumab," right?

A. That's correct.

Q. Hillmen tested for efficacy in treating PNH, right?

A. Yes, that's correct.

Q. And "Hillmen concluded that 'eculizumab is

safe and well tolerated in patients with PNH,'" right?

A. Yes, Hillmen concluded -- a direct quote from the paper -- that "Eculizumab is safe and well tolerated in patients with PNH," yes, that's correct.

Q. You don't have any reason to doubt that, right?

A. No.

Q. Going to paragraph 37 of your declaration, your '149 declaration, in this paragraph you're discussing the Hill 2005 paper, Amgen-1047, right?

A. Yes, that's correct.

Q. So it's your understanding that after the completion of the trial, the 11-patient trial that we saw reported in Hillmen, AMG-1004, the same 11 patients were enrolled in Hill '05's extension study in which the patients continued to receive eculizumab for a period of 52 weeks, right?

A. Yes, I see that in the declaration, and that's my understanding.

Q. And as Hill '05, AMG-1047, reported, long-term use of eculizumab in these patients resulted in sustained reductions of hemolysis and blood transfusions and continued improvement in

quality of life, right?

A. Yeah. Again, I see the sentence "Long-term complement inhibition with eculizumab in this patient population has resulted in sustained reductions in hemolysis and blood transfusions and continued improvement in life -- in quality of life," yes.

Q. That's your testimony in this case, right?

A. That's a direct quote from the article and it's also my testimony.

Q. All right. Fair enough.

And let's take a look at Hill '05 itself. Do you have that in front of you, AMG-1047?

A. Yes, I do.

Q. All right. Great. I have to get my copy.

So turn to the last page of AMG-1047. Do you see another journal cover page?

A. Yes, I do see this.

Q. Hill '05 was published in the journal Blood, right?

A. That's correct.

Q. Same journal as Tacken, right?

A. I'm sure that's right, yes.

Q. That's right, yeah.

Hill '05 was published in October of 2005,

right?

A. Yeah. It says the 1st of October 2005.

Q. Right. That's two months after Tacken's publication, right?

A. Let me look at Tacken just to refresh my memory. So Tacken was 15 August 2005.

Q. Right. So Hill 2005 is the later of the two publications between Tacken and Hill, right?

A. Yeah. I agree that October is after August, yes.

Q. All right. Fair enough.

Let's look at paragraph 38 of your declaration, your '149 declaration. Here you're discussing the Bell reference, AMG-1005, right?

A. Yes.

Q. You understand that Bell was another publication that disclosed methods of treating PNH patients with eculizumab, right?

A. Yes, that's correct.

Q. All right. Bell discloses data from the same studies with 11 patients that we saw reported in Hillmen, AMG-1004, and Hill, AMG-1047, right?

A. Yes, that's correct.

Q. And in -- in view of the results of those studies Bell concluded that eculizumab is, as you

say here, a particularly useful therapeutic for treating PNH, right?

A. Yeah. It says -- the direct line is "Bell concluded that eculizumab is a 'particularly useful' therapeutic for treating PNH."

Q. And that's your testimony in this case, right?

A. That's correct.

Q. In Paragraph 39 of your declaration, your '149 declaration, you discuss the Hillmen 2006 study, right?

A. That's correct.

MR. DIAMOND: Let's mark this. This is 1012. We'll figure it out later.

BY MR. DIAMOND:

Q. So you have in front of you, sir, the Hillmen 2006 paper, AMG-1012?

A. Yes, that's correct.

Q. And like Hillmen '04, Hillmen '06, AMG-1012 was published in the New England Journal of Medicine, right?

A. That's correct.

Q. Hillmen '06 was published on September 21st, 2006 if you look at the bottom of the front page here, right?

A. Yes, I see that.

Q. That's more than a year after the publication of Tacken, right?

A. Tacken was August from 2005. So that's correct, that's more than a year.

Q. Now, looking back at paragraph 39 of your declaration, your '149 declaration, you understand that -- that Hillmen 2006, AMG-1012, published the results of a larger phase 3 clinical study that was called the Triumph study, right?

A. Yes, that's correct.

Q. What is a phase 3 clinical study?

A. A phase 3 clinical study is a study that's conducted typically in a large patient population to make assessment of efficacy and toxicity typically done prior to the approval of a drug.

Q. And the Hillmen '06 phase 3 study described in AMG-1012, you understand that treated 87 patients with eculizumab over the course of 26 weeks?

A. Yes, I do understand that.

Q. All right.

And going on to page -- if you go to page 28 of your '149 declaration, here you have a section 6D regarding what was publicly known prior

to March 15th, 2007 about eculizumab's structure; do you see that?

A. Yes, I do see that.

Q. And if you look at paragraph 43 in this section, in the first sentence you cite to Hillmen '04, that's AMG-1004, and you cite to Hillmen '06, AMG-1012, as teaching that eculizumab is a humanized antibody, right?

A. Yeah. I'm sorry. You lost me for a second there when you said --

Q. Sure. I've taken you to paragraph 43 of your '149 declaration. Are you there?

A. So continuing on to page 39?

Q. I'm looking -- the paragraph starts on page 28 and bridges to page 29.

A. Yes.

Q. I'm focusing you on the very first sentence of that paragraph. Are you -- are you there?

A. Yeah. So the sentence that says --

Q. So what you --

THE REPORTER: Guys.

Q. Right. My question is if you look at that paragraph and that first sentence, you're citing to Hill '04 and you're citing to Hill '06 -- I'm

sorry. Let me rephrase that.

In the first sentence in paragraph 43 of your '149 declaration you're citing to Hillmen 2004 and Hillmen 2006 for the statement that eculizumab is a humanized antibody, right?

A. Let's see. So the reference is to AMG-1004 and that is Hillmen from 2004 and to AMG-1012 and that reference is Hillmen from 2006.

Q. Right. So just going -- now that you've had a chance to check that out, going back to my question. In the -- in the first sentence of -- of paragraph 43 of your '149 Patent declaration you're citing Hillmen '04 and Hillmen '06 as teaching that eculizumab is a humanized antibody, right?

A. Yes. So eculizumab is a humanized antibody --

Q. Right.

A. -- and those two references are cited, yes.

Q. Great.

So you agree that Hillmen 2004 and Hillmen 2006 are at least two references that a POSA prior to March 15th, 2007 would have considered for an understanding of what eculizumab is, right?

A. I -- I think that they would have considered these articles with respect to eculizumab's efficacy as we've talked about, yes.

Q. Okay. And considered it with the understanding that eculizumab is a humanized antibody, right?

A. Yes. This comes directly from the references, correct.

Q. Great.

Further on in paragraph 43 you also cite to Bell, AMG-1005; do you see that?

A. Yes, I do see a reference to Bell.

Q. Yeah. Let's actually look at paragraph 70 of your '149 declaration.

A. I'm sorry. Paragraph 70?

Q. 70, 7-0, yeah.

A. Okay.

Q. So here you're offering your opinion in this section 10 of your '149 declaration beginning at paragraph 70, you're offering your opinion that claim 1 of the '149 Patent was anticipated by Hillmen, right?

A. Yes, that's correct.

Q. And in paragraph 71 you're offering your opinion here that Hillmen discloses claim 1's

limitation of an antibody that binds C5, right?

MS. STERLING: Objection, form.

A. So the reference here is to AMG-1001, 39:2.

Q. That's claim 1 of the patent, right?

A. Okay. Yeah.

Q. This is really simple. You're saying here in this paragraph that it's your opinion that Hillmen discloses claim 1's limitation of an antibody that binds C5, right?

A. Yeah. I -- I --

Q. That's what it says there, right?

A. Yes. It says in the declaration similarly "Hillmen states that eculizumab 'binds specifically to the terminal complement portion of protein C5.'"

Q. I was asking you a simple question. I was just asking you to confirm that you said something in the declaration.

So you agree with me that Hillmen -- you say that "Hillmen discloses claim 1's limitation of an 'antibody that binds C5,'" right? Do you say that?

A. Yeah. I'm going to apologize for a second that you're asking questions quickly and I just want to be sure that I'm answering in a manner

that's completely accurate --

Q. Yeah, and I think --

A. -- and so I'm referring to the declaration.

THE REPORTER: Guys.

Q. Yeah. I -- I -- so I was just reading a sentence out of your declaration. You say in your declaration "Hillmen discloses claim 1's limitation of an 'antibody that binds C5,'" right, sir? Do you see that?

A. I do see that sentence within the declaration, yes.

Q. Great. That's part of your testimony, right?

A. That's correct.

Q. All right. Great.

THE REPORTER: You have to slow down.

MR. DIAMOND: We'll do our best here, but we've got to keep going.

THE REPORTER: You know what? I'm sorry. I have to take a break.

THE VIDEOGRAPHER: Going off the record?

MS. STERLING: It appears so.

THE VIDEOGRAPHER: Going of the record. The time is 12:49.

(A short break was had.)

THE VIDEOGRAPHER: Going back on the record. The time is 12:52.

BY MR. DIAMOND:

Q. Dr. Balthasar, are you still at paragraph 71 of your '149 Patent declaration?

A. Yes, I am.

Q. Great. If you look at the -- the last full sentence on page 47, you see it states that "Eculizumab binds specifically to the terminal complement protein C5"?

A. Yes, I see that.

Q. And you are citing Hillmen '04, AMG-1004 at page 553 for that, right?

A. Yes. That's the citation that's listed.

Q. That's the citation that you list, right?

A. That's correct.

Q. All right. Let's -- let's look at -- I see that you've picked up something. Let's look at Hillmen at that page, 553. Let me get mine here. So if you turn to page 553 of Hillmen and you look at the paragraph just above the header for "Methods" in the left-hand column, do you see that paragraph beginning "Eculizumab is"?

A. Yes, I see that paragraph.

Q. All right. The sentence -- and going back to your '149 declaration, paragraph 71, the sentence that we read that "Hillmen states that eculizumab binds specifically to the terminal complement protein of C5," you're quoting from Hillmen '04 at page 553 in that statement, right?

A. Yes, that's correct. The direct quote is from that paragraph that you mentioned, it's the second --

Q. And would you please read the direct quote to us?

A. Yes.

Q. Thank you.

A. It's the second sentence and it reads "It binds specifically to the terminal complement protein C5, inhibiting its cleavage into C5a and C5b, thereby preventing the release of the inflammatory mediator C5a and the formation of the cytolytic pore C5b-C9."

Q. And -- and when that sentence that you just read and that you quote from in paragraph 71 of your '149 declaration, when it says "It binds specifically," what does the word "it" refer to?

A. So my understanding from reading this is that "it" is referring to eculizumab, which is

referred to in the prior sentence as "Eculizumab is a recombinant humanized monoclonal antibody."

Q. And could you -- well, I'll read to you that the prior sentence that you just mentioned reads in full "Eculizumab is a recombinant humanized monoclonal antibody that was designed to block the activity of terminal complement components."

MS. STERLING: Objection, form.

BY MR. DIAMOND:

Q. I'm sorry. I misread it. I'm going to read -- I'm going to reread it.

The statement -- the sentence prior to the one that you quoted in paragraph 71 of your '149 declaration reads "Eculizumab is a recombinant humanized monoclonal antibody that was designed to block the activation of terminal complement components," right?

A. That's correct.

Q. That sentence has two references, No. 14 and No. 15, right?

A. That's correct.

Q. And if you go to the references section of Hillmen '04, AMG-1004, which appears at page 559 of that document. Let me know when you're there.

A. I'm there.

Q. All right. Reference 15 is the Thomas paper that we've been looking at today, AMG-1023, right?

A. That's correct.

Q. And reference 14 is a Riechman, et al., paper regarding reshaping human antibodies for therapy from Nature 1988?

A. That's correct.

Q. Are you familiar with the Riechman paper?

A. Yes, I am.

Q. And that's -- that's just like a classic paper regarding humanization of -- of antibodies generally, right?

A. Yeah. I think both are papers that are -- talk about methods of humanization. So Thomas also talks about methods of humanization, Riechman talks about methods of humanization as well, yes.

Q. Fair enough. Riechman, as far as you know, wasn't talking about 5G1.1, right?

A. That's correct.

Q. But Thomas was, right?

A. Yeah. Thomas is talking about humanization of anti-C5 antibodies in single-chain Fe's, yes.

Q. And specifically designing an h5G1.1 humanized antibody?

A. It talks about humanization of 5G1.1, yes.

Q. Okay.

Let's look at paragraph 78 of your '149 declaration. Actually if we could look at -- look at paragraph 79. I think that's fine. Here in paragraph 79 you're discussing the Hill '05 reference, AMG-1047, right?

A. That's correct.

Q. Yeah. And you state that "Hill '05 discloses claim 1's limitation of an 'antibody that binds C5,'" right?

A. Yes, that's correct.

Q. You say that "Hill '05 discloses that eculizumab is an anti-5 -- anti-C5 antibody"; do you see that?

A. I see that in the declaration, yes.

Q. All right. To read the full sentence, you say "Hill '05 discloses that eculizumab is an anti-C5 antibody, characterizing it as a 'humanized monoclonal antibody that specifically targets the complement protein C5'"; do you see that?

A. I do.

Q. And in that sentence you're quoting from

Hill '05, AMG-1047, at page 2559, right?

A. That's correct.

Q. All right. So why don't you pull out Hill '05 that's in front of you and let me know when you're on that page, AMG-2559.

A. I'm on that page.

Q. Great. So I think you can find the complete sentence that you are quoting from in paragraph 79 of your '149 Patent declaration if you look at page 2559 of Hill '05 on the right-hand side in the first full paragraph, the sentence beginning "Eculizumab is." Are you there?

A. Yes, I'm there.

Q. All right. And you -- and you see that this is the sentence you were quoting from in the -- in the second sentence that we just read from paragraph 79 of your '149 Patent declaration, right?

A. Yes, that's right.

Q. The sentence in Hill '05 that you quote reads in full "Eculizumab is a humanized monoclonal antibody that specifically targets the complement protein C5 and prevents its cleavage," right?

A. That's correct.

Q. And that sentence in Hill '05 regarding

what eculizumab is cites to a reference No. 9; do you see that?

A. I do see that.

Q. And if we turn to the references section of Hill '05, which appears on page 2565 of that document, you see that reference No. 9 is the Thomas 1996 paper, which is AMG-1023 in these proceedings, right?

A. Yes, I do see that Thomas is referred to as reference No. 9. It's, again, the key paper that talks about humanization of these anti-C5 antibodies.

Q. Let's take a closer look at Hillmen 2006, which is AMG-1012. Do you have that?

A. I'm sure that I do. It will just take me a second. Yes, I do have that.

Q. All right. Look at the last -- so go to page 1234, that's the first page on the inside, and if you look on the left-hand side towards the middle of the page, it's the last full paragraph on that column beginning with "Eculizumab (Soliris)"; do you see that?

A. I do see that.

Q. All right. That full sentence reads "Eculizumab (Soliris, Alexion Pharmaceuticals) is a

humanized monoclonal antibody directed against the terminal complement protein C5"; do you see that?

A. I do see that sentence.

Q. So this sentence is talking about what eculizumab is, but it's also talking about what -- what Soliris is, right?

A. I see the sentence that it references eculizumab and Soliris, yes.

Q. Right.

Now, a POSA reading this Hillmen 2006 paper would have understood that Soliris was -- was Alexion's proposed brand name for the eculizumab monoclonal antibody, right?

A. Yes, I think that's correct.

Q. The sentence that we just read talking about what eculizumab and Soliris is, it cites to a reference No. 13; do you see that?

A. I do see that.

Q. And reference 13, if you go to page 1243 of this document, AMG-1012, reference 13 is the Thomas paper that we've seen as AMG-1023, right?

A. Right. They're referencing Thomas, this key paper talking about humanization of 5G1.1 antibodies, yeah.

Q. So they cite Thomas, right?

A. It's cited as the reference, yes.

Q. Okay.

There's nothing in Hillmen '04, Hill '05, or Hillmen '06 that you have in front of you, AMG-1004, 1047 and 1012, there's nothing in those references that describes eculizumab or Soliris as having an IgG2/IgG4 hybrid constant region, right?

MS. STERLING: Objection, form.

A. So the only reference that I would make to eculizumab as having that structure is in that the use of eculizumab directs one to prior information about the structure of eculizumab --

Q. Sir, my question's a little bit different.

A. -- and prior knowledge --

Q. I'm asking you about what's actually in these three documents. Okay?

A. Uh-huh.

Q. So you've got them in front of you. You've got AMG-1004, AMG-1047, and AMG-1012 in front of you, correct?

A. Correct.

Q. All right. So in those three documents in front of you right now there's nothing in them that describes eculizumab or Soliris as having an IgG2/IgG4 hybrid constant region, right?

MS. STERLING: Same objection.

A. Yeah. I think that you're saying is there any direct indication saying G2/G4 within these papers and --

Q. My -- my question is -- I'll ask my question again because I think it's straightforward. You've got those three documents in front of you, Hillmen '04, Hillmen '06 and Hill '05, and there's nothing in those documents that you could point me to right now sitting here today that states that eculizumab or Soliris has a hybrid IgG2/G4 constant region, right?

MS. STERLING: Same objections.

A. So I'd like to -- so my question is that the name eculizumab is assigned to an antibody --

Q. Sir, sir, that's not -- that's not my question.

MS. STERLING: Excuse me, Counsel --

MR. DIAMOND: I didn't ask you -- no, no. He's not answering my question.

MS. STERLING: You've inter- -- you're letting him say three words and then you're interrupting him.

THE WITNESS: Yes. He's --

BY MR. DIAMOND:

Q. Sir, this is -- this is my one opportunity, sir, to talk to you about your opinions in this case, okay, and you're here today to give me the testimony that's going to be before the Board. Okay. So I need you to listen to my questions and I need you to answer my questions, and my question is very simple. It's about those three documents. It's not about the --

A. Can I explain the confusion?

Q. It's not about -- sir, it's not about -- it's not about -- it's not about what you think the word eculizumab -- I'm asking about what is stated in those documents. If you can point me to something in those documents, let me know, point me to it, please, but my question to you is you cannot point me to anything in those three documents where it states in words that eculizumab or Soliris has an IgG2/G4 constant region or where it cites another reference that would indicate that, right?

MS. STERLING: Objection, form.

MR. DIAMOND: If you can point to something, please point to me. If you can't point to me -- point me to something, then say you can't.

MS. STERLING: Objection, form.

A. So I -- I will answer your question, but I

just want to first explain --

Q. No, sir.

A. -- what my confusion is.

Q. No, sir. You will answer my question. Okay. If your counsel has questions for you later, she can ask you to explain whatever it is that you have in your head, but right now my question to you is about what is in the four corners of these documents, including the references, sir. So if you think that there's a reference in one of those three papers that talks about eculizumab or Soliris having an IgG2/G4 constant region, then point me to that reference. Or if you think there's words in the paper that state that, then point me to that. And if you can't point me to that in any of those three references, Hillmen 2004, Hillmen 2006 and Hill 2005, then say that, but you have to answer my question, sir.

A. Yeah. So if the question is do I see anywhere within these three references mention of G2/G4, the answer is no --

Q. Okay. And then the next question --

A. -- but eculizumab --

Q. Sir, that's -- that's one question.

A. -- is an antibody --

Q. The next question is --

MS. STERLING: I'm sorry.

A. -- that is known to have sequence --

Q. Sir, sir, sir --

A. -- of G2/G4.

MS. STERLING: I'm sorry. For the sake of the record, can we stop talking over each other.

MR. DIAMOND: For the sake of the record, this witness has to answer my questions. He answered my question.

BY MR. DIAMOND:

Q. The next question is in these three references is there any -- okay.

Well, in these three documents, Hillmen 2004, Hillmen 2006 and Hill 2005, is there any reference in the references cited, right -- you see references Nos. 1 through 21, for example, in Hillmen 2006? Do any of those references, to your knowledge, state that eculizumab or Soliris has an IgG2/G4 constant region? And if the answer is no, then say no.

A. Am I allowed to explain my answers? I thought that I was allowed to explain my answers.

Q. No. You're required to give an answer.

A. Uh-huh.

Q. And by offering an explanation without an answer you've got no answer. So I need --

A. In the last question I gave an answer --

Q. No, sir --

A. -- and was continuing to explain it --

Q. Sir, no.

A. -- and you interrupted me and talked over me.

Q. Sir, you answered --

A. Am I allowed to give an explanation?

Q. No. You're not allowed to answer -- ask yourself your own questions. You have to listen to my question, and my question is -- okay. Let's take this one by one. Take out Hillmen 2006. Do you have that in front of you, AMG-1012?

A. Yes, I do.

Q. Yes. Okay. Go to page 1243. Okay? Are you there?

A. Yes.

Q. This is the references section for Hillmen 2006, right?

A. Yes, I see that.

Q. There are 21 references listed in Hillmen 2006, right?

A. Yes, that's correct.

Q. To your knowledge sitting here today, do any of these 21 references in Hillmen 2006 state that eculizumab or Soliris has a humanized IgG2/G4 heavy chain constant region?

A. I have to admit I haven't read through every one of these references to know.

Q. So you don't know?

A. I haven't read through them to know whether they do or do not.

Q. So sitting here today, the answer is I don't know?

A. So sitting here today, the answer is I have not read through these to be able to know if they do indicate anything about a G2/G4 domain.

Q. Do you know?

A. I just said sitting here today, I do not know if any of these references give any indication to a G2/G4 domain.

Q. Fair enough.

A. Many of these references I haven't read.

Q. Is the same true with respect to the references if we take Hill 2005, AMG-1047, that sitting here today, you don't know if any of the 25 references listed on page 2565 of Hill '05 identifies eculizumab or Soliris as having an

IgG2/G4 heavy chain constant region?

(Witness reviewing document.)

A. So after looking through the titles of all the references, my answer is very similar to that which I just gave, that I do not know if any of these papers make reference to a G2/G4 domain of eculizumab.

Q. Okay.

A. They may make reference to a G2/G4 domain and they may not.

Q. You don't know.

So if you go to -- let's just confirm the same thing for AMG-1004 and the references listed there on page 539. So you see there is 25 references listed on page 539 of AMG-1004, right?

A. Yes. I do see 25 listed, yes.

Q. And same question. Sitting here today, you don't know whether any of these 25 references identifies eculizumab or Soliris as having a IgG2/G4 heavy chain constant region, right?

(Witness reviewing document.)

A. Yes. I've read through all of the author lists, the title, reference information, and, as you know, similar to the responses I gave for the

last two publications, there's many articles here that I've not read and don't know the entire contents of. And so I do not have any knowledge of whether or not they have reference to eculizumab's G2/G4 domain. They may and they may not. I just don't know.

Q. So you don't know.

Now that you've had a chance to look through the references, though, of Hillmen '04, Hill '05, and Hillmen '06, they don't cite the Bowdish, right?

A. I did not see the -- see the Bowdish patent do you mean, or --

Q. They don't cite to Bowdish which is, you know, AMG-1006 that you talk about in this case as the grounds of some of your -- some of your anticipation and obviousness positions? They don't -- they don't cite to Bowdish, right?

A. Yeah. Let me just look at my reference list to be sure that I know what you're referring to.

Q. Sure. So it's, yeah, AMG --

A. 1006.

THE REPORTER: Guys.

Q. -- 1006.

A. So the 100 --
Q. So using that definition of Bowdish, yeah.
A. So the 1006 exhibit refers to a U.S. patent application, and it's correct that it's not referred to in any of these documents.
Q. Okay.
A. And, again, it would be unusual to see a patent reference within a publication of that type.
Q. Okay. And just -- why don't you stick with your index of documents there just so I can refer to things easily.
A. Sure.
Q. But do you see there's a -- there's a Mueller reference we looked at earlier, which is AMG-1008, right?
A. Yes, I see that.
Q. There's a Mueller 2 reference, which is AMG-1031; do you see that?
A. Yes.
Q. Mueller 2 is a -- it's a published article upon the patent, right?
A. Yes, that's correct.
Q. And based upon the -- the review that you just did of -- of the references for each of Hillmen 2004, Hillmen 2006, and Hill 2005, you

didn't see any references to either of those two Mueller documents, right?

A. So those papers -- I have to admit I -- I don't know that I was specifically looking for Mueller, but --

Q. It would have jumped out to you if you saw Mueller, right?

A. Maybe, maybe not. I'd rather look through them again to be sure.

Q. Let me just ask -- I don't want to -- we have a lot to go through here, but in your -- in your review you sat there before and you looked at all the references to see if there was anything that you thought related to IgG2/G4 in eculizumab. You didn't notice the Mueller references when you were reading through the references just now, right?

A. So when I went through the list I -- I didn't notice the Mueller references, correct.

Q. All right. Were there any particular references you were looking for when you were doing that review?

A. Just looking through the list, looking through the titles to be able to see if there was any reference to G2/G4.

Q. And when you were looking through I take it you didn't see -- sorry. When you were looking through those three documents, Hillmen 2004, Hillmen 2006 and Hill '05 in the references section, you didn't see any reference to Tacken, right?

A. So I -- I did not see any reference to Tacken as I was scanning through, no.

Q. Okay. And you -- and you didn't see any -- if you refer to Evans, which is AMG-1007 in your list of documents there, and just refresh yourself with what Evans is. It's a patent. Okay? Do you see that?

A. I do see that, yeah.

Q. All right. It's -- it's a patent ending in the No. 245, right?

A. Correct.

Q. You didn't see any reference from your review just now of all the references in Hillmen 2004, Hill 2005, and Hillmen 2006 to the Evans patent, right?

A. Yeah. In going through the list of references through the documents, right? I didn't see any patents listed. And that would be unusual. This patent I didn't notice as being present in the

reference list.

Q. Earlier you mentioned I think when we were talking about Thomas, you said there was a difference between certain fragments, like an scFv and an intact antibody; is that right?

A. Yes, an scFv antibody is a fragment of an -- of an intact antibody, I guess, yes.

Q. And -- so the F in scFv stands for fragment, right?

A. That's correct.

Q. And the V stands for vari- -- variable region?

A. Yes.

Q. And scFv is -- it's two variable regions connected that are then connected by a link or in a single chain, right?

A. That's correct.

Q. And -- and that's not -- an scFv is not something that's like physically or chemically fragmented off an antibody. It's something that's developed by recombinant DNA technology, right?

A. That's correct.

Q. And scFv doesn't have a heavy chain constant region, right?

A. That's correct.

Q. All right. Let's talk a bit more about Bell. So you rely on -- on Bell, AMG-1005 -- yeah. You have -- you have Bell, AMG-1005, in front of you, right? I want to make sure I gave it to you.

A. Yes, I do have it.

Q. Great. And you rely on Bell in support of a number of your obviousness grounds in your three declarations in these proceedings, right?

A. Yes. We do make citation to this document within the declaration in many places, yes.

Q. You more than cite to it. It's one of the references that are the basis of some of your obviousness (inaudible), correct?

A. That's correct, yes.

Q. For example, you combine Bell with the Bowdish and Evans references, right?

A. That's correct.

Q. You combine Bell with the Mueller and Evans references, right?

A. That's correct.

Q. Now, you understand that Bell doesn't disclose the element in the challenged claims that the claimed antibody comprises a heavy chain consisting of Sequence ID No. 2 and a light chain consisting of Sequence ID No. 4, right?

A. I'm sorry. Could you repeat the question.

Q. Maybe it's just easier if I take you to your declaration. So if you look at your '149 declaration at paragraph 105. So if you look at this -- are you there, paragraph 105?

A. Yes.

Q. If you look at the second sentence, you say "Bell teaches" -- sorry. The second sentence of that paragraph you say "Bell teaches 'an antibody that binds C5' as claimed because it teaches treating PNH with a pharmaceutical composition comprising the anti-C5 antibody eculizumab," right?

A. That's correct.

Q. And then in the next sentence you say "The only element of Claim 1 not expressly described in Bell is the requirement that the anti-C5 antibody 'comprises a heavy chain consisting of Sequence ID No. 2 and a light chain consisting of Sequence ID No. 4'"; do you see that?

A. I do see that statement.

Q. Yeah, And then you have your '504 declaration in front of you?

A. Yes, I do.

Q. So if you go to paragraph 172 of your '504

declaration --

A. Yes, I'm there.

Q. -- second sentence you say that "Bell teaches methods of treating PNH with a pharmaceutical composition comprising the anti-C5 antibody eculizumab," right?

A. Yes, I do see that.

Q. But you say "The only element of the challenged claims not taught in Bell is that the anti-C5 antibody 'comprises a heavy chain consisting of Sequence ID No. 2 and a -- and a light chain consisting of Sequence ID No. 4,'" right?

A. I see that sentence, yes.

Q. So I just want to make sure I understand your testimony here. With respect to Bell, just taking the Bell reference, you understand that Bell doesn't teach the element of an anti-C5 antibody that comprises a heavy chain consisting of Sequence ID No. 2 and a light chain consisting of Sequence ID No. 4, right?

A. So yes, I have stated that in the declaration --

Q. Okay.

A. -- that it's not taught by Bell, that --

Q. Okay. So to get to -- to get to Sequence ID No. 2 and Sequence ID No. 4 as recited in the challenged claims you combine Bell's teachings with amino acid sequences that you take from other references, some sequences from -- from Evans and then amino acid sequences that you take from either the Bowdish reference or the Mueller reference, right?

A. That's correct.

Q. Okay. There's nothing in Bell, which you have in front of you there, AMG-1005, that discloses that eculizumab has a hybrid IgG2/IgG4 heavy chain constant region, right?

A. Again, I reviewed Bell and have a fair bit of confidence in what's in Bell, but I wouldn't want to make a statement like that without reviewing word for word, line by line. So I'm mostly referring to Bell with respect to method of use issues.

Q. Well, let's just make this clear. Sitting here today, you can't point to me anywhere in Bell where it discloses that eculizumab has an IgG2/IgG4 heavy chain constant region, right?

A. I don't know if I'd be able to do that or not without reading Bell word for word and line by

line.

Q. Okay. But with the knowledge that you have for Bell from your preparation to give us testimony here today --

A. Yes.

Q. -- and all your review and preparation that you put into these declarations, sitting here today right now, you can't point anywhere in Bell to show me where Bell identifies eculizumab as having an IgG2/G4 constant region, right?

A. Yeah. So again, I'm not trying to make any statement about whether or not there's any indication there, but I would say that you're correct that right now I'm not able to just pull up a section and point to that particular reference.

Q. Sir, today -- as I said, today is the day that you are here to give testimony, all right, that's going to be before the Board on your opinions. So we need to understand what is in these references and what's not. I mean, you're offering opinions in this case about what's in these references and what's taught by the art and what's not.

So I'm asking you, sir, can you tell me whether Bell -- anywhere in Bell teaches that

eculizumab has an IgG4 -- IgG2/IgG4 heavy chain constant region?

A. Yeah. So I don't have complete confidence in the entire document to know it line by line and word by word to be able to say that. You asked the question can I point to something right now, and my answer to that is without saying whether or not something exists I can tell you that I don't have the ability to point to that particular reference at this particular moment.

Q. So you don't -- your testimony here today is -- for the Board is that you don't have confidence as to say what Bell does and doesn't disclose with respect to this case?

MS. STERLING: Objection, misstates the witness's testimony.

THE WITNESS: Yeah --

MR. DIAMOND: I think accurately stated his testimony.

MS. STERLING: I think if we read it back you'll be wrong.

A. I did say that the reason why I referred to Bell is in terms of method of use.

Q. Sir, that's not my question. My question is --

A. But that's what my testimony relates to.

Q. Your testimony just now is that you have no con- -- you -- you -- you -- the testimony you're offering for this case and for the Board is that you have no confidence as to your understanding of whether Bell does or doesn't disclose that eculizumab has an IgG2/G4 constant region, right?

MS. STERLING: Objection, misstates the witness's testimony.

A. You've made a specific request about a specific statement, and my response to that is I can't say at this particular moment that I have a word-for-word recollection of Bell to be able to say yes or no G2/G4 is listed within Bell. So --

Q. It's your -- it's your opinion in this case, sir, that eculizumab --

A. I have -- so I will say this. I have not cited within my declaration a reference to Bell as indicating a G2/G4 domain.

Q. And you don't plan to offer that opinion in this case, right?

MS. STERLING: Objection, relevance.

A. I don't plan to give additional information, as we talked about earlier in the

lead-up to this deposition, to offer additional things beyond what I've stated in the declaration.

Q. Fair enough. So at least we can -- we could agree that you're not going to offer any opinion in this case that Bell discloses that eculizumab has an IgG2/IgG4 heavy chain constant region, right?

A. Yeah. I can agree that I don't have any statement about Bell offering data or reference to a G2/G4 domain within my declaration, and I don't expect to give any additional information beyond what we talk about today.

Q. Okay.

If you look at paragraph 107 of -- going back to your '149 declaration, in the very last sentence of this paragraph --

A. I'm sorry. I was just going to the '149 declaration. What paragraph?

Q. Yeah. The '149 declaration, it's on page 68 of your '149 declaration, and it's paragraph 107.

A. Okay.

Q. Let me know when you're there.

A. I'm there.

Q. All right. Great. So I would direct you

to the bottom of the page, last sentence. You say "Therefore Bell discloses 'an antibody that binds C5' as required by claim 1 in its disclosure of eculizumab and provides a reason to make that antibody," right?

A. Yes, that's my testimony.

Q. Okay. So the reason you're relying on Bell here is because Bell discloses the element of an antibody that binds C5, which is an element that's required by claim 1 of the '149 Patent, right?

MS. STERLING: Objection, form.

A. So I agree that this sentence says that "Bell discloses 'an antibody that binds C5' as required by claim 1 in its disclosure of eculizumab and provides the reason to make that antibody." That is exactly my testimony. You're exactly correct.

Q. And the antibody that binds C5 that's disclosed by Bell is eculizumab, right?

A. Yes, that's correct.

Q. Okay. Bell actually uses that name eculizumab to refer to that C5 binding antibody, right?

A. That's correct.

Q. Yeah. And as you explain at the top of the paragraph here, you say that Bell -- Bell taught -- or Bell disclosed that eculizumab was an anti-C5 antibody that blocks the conversion of C5 into complement component C5a and C5b; do you see that?

A. Yes, that's part of my testimony.

Q. Yeah. And that's something that Bell would have taught a POSA prior to March 15th, 2007, right?

A. Yes. I think a POSA could read that section directly from Bell, that's a direct quote from Bell.

Q. Right. Well, that's why you're -- you're offering all this information as information that a POSA prior to March 15th, 2007 would consider, right?

A. Well, I mean, I'm offering it with respect to a particular argument, but yes, this is something that a POSA would be able to know and appreciate.

Q. All right. And Bell includes data from clinical studies showing that eculizumab could be used to treat PNH, right?

A. That's correct.

Q. Now, it's your opinion that a POSA as of March 15th, 2007 would have been motivated by the teachings in Bell and the other clinical references that we saw, like Hillmen 2004 and Hill 2005, to make and use the eculizumab antibody that they describe, right?

A. Yes, I do believe that.

Q. Well, let's take a look at page 20 -- actually, no. Why don't we go into Bell itself. So take out AMG-1005, which is in my stack somewhere. Maybe it's not. There it is.

While I'm finding my copy why don't you turn yourself to paragraph 52, and let me know when you're there. It's on the page with the page No. 17 at the bottom.

A. Yes, I'm at paragraph 52.

Q. Great.

So you've relied on this paragraph 52 of Bell in these proceedings, right?

A. Yes, I'm sure that's correct.

Q. Yeah. So if you look at the left-hand column on this page in paragraph 52, about four lines up from the bottom you see a sentence beginning "Particularly useful"?

A. Yes, I do see that.

Q. So Bell says "Particularly useful anti-C5 antibodies are hh5G1.1-mAb, h5G1.1-scFv, and other functional fragments of h5G1.1"; do you see that?

A. Yes, I do see that.

Q. So Bell's referring to at least three different structures in this sentence, one being h5G1.1-mAb, another being h5G1.1-scFv, and then, third, other functional fragments of h5G1.1, right?

A. I don't know that I would say that that's necessarily correct. So I would look at the designation of h5G1.1-mAb, h5G1.1-scFv, and the reference of other anti-C5 antibodies that are fragments of h5G1.1 as representing possible families of compounds rather than just three specific constructs.

Q. Okay. And so -- I'm not disagreeing with you. I think I said that Bell is referencing at least -- at least three different structures, right? Possibly more?

A. So I could agree with at least three.

Q. At least three and possibly more?

A. At least three, yeah.

Q. And -- and maybe it will be clearer if I just say a person of skill in the art reading Bell would understand that h5G1.1-mAb is different from

h5G1.1-scFv, right?

A. Yes.

Q. And that a person of skill in the art would also have understood that both of those are different from whatever the other functional fragments of h5G1.1 that are identified here, right?

A. Yes, I would agree.

Q. Okay. So the h5G1.1-scFv as we discussed earlier, that's a single chain variable region fragment comprising two variable regions, a variable heavy region and a variable light region connected by a linker, right?

A. That's the structure of an scFv, yes.

Q. Right.

A. So, again, when you said "a," I don't -- I would like to indicate this refers kind of like to a family versus to a single entity.

Q. Well, if we go to -- if we go to paragraph 12 of Bell, AMG-1005, which is on the bottom page No. 13.

A. Yes.

Q. Are you there? And the last sentence there that it bridges onto the next page, but you see it says "In particularly useful embodiments the

compound is an anti-C5 antibody selected from the group consisting of h5G1.1-mAb (eculizumab), h5G1.1-scFv (pexelizumab), and other functional fragments of h5G1.1," right?

A. I see that sentence, yes.

Q. So a person reading this would understand that the h5G1.1-scFv here in Bell has been given a specific name, pexelizumab, right?

A. So I think that a person of ordinary skill in the art would see that pexelizumab is an example of an scFv that has this characteristic of being an h5G1.1-scFv. So there could be many. Pexelizumab is an example of one. It's one that's being specifically referred to here.

Q. And this sentence doesn't say pexelizumab is an example, right?

A. Correct.

Q. That's something you're reading into this?

A. It's more just from the nomenclature that these designations of scFv or mAb are generic designations that aren't referring to a specific entity. So eculizumab and pexelizumab, as we talked about before, as named entities are a particular entity that have in this case the structure of either an scFv or a mAb.

Q. Let's go back to -- well, let me ask this. A POSA would understand that as compared to scFv's, when this refers to h5G1.1-mAb, an h5G1.1-mAb antibody would be a full-length intact humanized antibody, right?

A. Yeah. I think that that would be the general appreciation of a person of ordinary skill in the art.

Q. Okay. And --

A. I should --

Q. I'm sorry.

A. -- qualify that a little bit in that even if you look at approved antibodies, there's a number of fragments that are referred to as mAb's. So there's a number of examples where people say that -- for example, eb6mAb (phonetic) is a mAb and it's a Fab fragment. So there is a little bit -- it has to be within the context I guess of use. And so I think a POSA would certainly understand that a Fab is not an intact antibody and typically associate mAb with an intact antibody, but there's examples where the term mAb has been used to define things that include mAb fragments.

Q. But Bell is differentiating -- no, let me -- let me rephrase that.

So it's your -- it's your opinion that if a person of skill in the art sees the phrase "h5G1.1-mAb" they're not certain what that's referring to?

A. Yeah. I think that that's fair. I mean, h5G1.1-mAb would be referring to -- I think most POSA's would indicate that this refers to a humanized 5G1.1 antibody, that is, a full-length, and that it may relate to multiple entities that fit that description. The qualification in parentheses here of referring to eculizumab refers, again, to a specific example of this particular type of name compound.

Q. Well, why don't we go back to paragraph 52 of Bell. So at the end of that paragraph, which is on the right-hand side towards the top, the last sentence you see Bell states that "The antibody h5G1.1-mAb is currently undergoing clinical trials under the trade name eculizumab," right?

A. Yeah. I do see that sentence, yes.

Q. All right. And -- and that -- that would refer to a full-length intact humanized antibody, right?

A. So, you know, everything I guess you would want to understand in context, and I agree, yes,

that h5G1.1-mAb is referring to an intact antibody through its linkage here in this sentence to eculizumab --

Q. Okay.

A. -- Which is an intact G2/G4 antibody that's humanized.

Q. But as we talked about, Bell doesn't refer to the G2/G4 structure at all, right?

A. Yeah, that's correct. Again, the reason why I waffle on that a little bit is that an antibody is defined by its structure --

Q. Sir, that was -- that was my question. Again, your counsel is free to ask you questions about -- she's not free to talk to you about it during the deposition, but she's free to ask you questions to follow up on anything towards the end of deposition. So I'm going to ask my questions.

But if we look at -- sticking with paragraph 52, you see the next sentence down in the left-hand column from what we were reading says "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 -- and then it cites a reference which you recognize is -- is the Thomas reference we've been

looking at, right?

A. Yes. Correct.

Q. And you recognize the -- that the patent number that was cited there, 6,355,245, that's the Evans patent, which is AMG-1007 in these proceedings, right?

A. Correct.

Q. Let's -- and those are the -- I guess Evans and Thomas are the only two references that Bell cites for methods for the preparation of h5G1.1-mAb, h5G1.1-scFv, and other functional fragments, right?

A. Yeah. I think it's correct that those two references are cited as giving general information about methods that would be suitable for preparation, yes.

Q. That -- that wasn't my question. It doesn't say -- you said general methods. That doesn't say general methods there, does it?

A. It says "Methods for preparation" in the beginning of that sentence, and that's what I referred to.

Q. And those -- those are the two references -- the only two references that are cited for that proposition, right?

A. Those two references give information about the methods that can be used for preparing humanized versions of C5 antibodies of the G1.1.

Q. Uh-huh.

A. Yep.

Q. And it doesn't cite any other references for that proposition, right?

A. No. I just see citation for those two publications for --

Q. Just Evans and Thomas?

A. -- for methods.

Q. Okay.

MR. DIAMOND: Why don't we mark that as 1007.

THE REPORTER: I'm going to need a break soon.

MR. DIAMOND: Yeah. Sure. Let's go ahead and do this quickly, AMG-2007.

BY MR. DIAMOND:

Q. So you're being handed -- so you have AMG-1007 in front of you. That's the Evans reference that you cite in these proceedings, right?

A. That's correct.

Q. And this -- this is the Evans reference

that you cite as one of the -- one of the bases for a number of your obviousness contentions in these proceedings, right?

A. That's correct.

Q. Now, Evans discloses how to make humanized 5G1.1-scFv, right?

A. There's some description for scFv's, yes.

Q. And Evans also describes some other functional fragments like Fd's and light chains, right?

A. That's correct.

Q. But Evans doesn't describe any -- how to make any full-length humanized 5G1.1 antibody, right?

A. I have to say not to my best recollection.

Q. Okay.

A. Yeah. I don't recall specific reference to that offhand.

Q. Okay. Thomas does disclose the preparation of a full-length h5G1.1 humanized monoclonal antibody, right?

A. Yeah. Thomas gives description of methods to humanized antibodies and gives an example of that, yes.

Q. Okay. Nothing in Evans, AMG-1007,

describes or suggests using a humanized antibody specifically with a hybrid IgG2/IgG4 isotype, right?

A. So I don't think I've cited anything like that within my declaration for Evans.

Q. Okay.

A. That's, you know, consistent with my best recollection of Evans. Without going through it line for line I wouldn't want to make that type of statement.

Q. Well, I'm asking you about your testimony in this case and why you're relying on Evans. As you're sitting here right now, that's your understanding that Evans doesn't disclose that, right?

A. Yeah. I'm relying on Evans really for the provision of information that relates to CDR's and variable fragments of the 5G1.1 antibody derivatives.

Q. Okay. And CDR's are portions of the variable sequence, right?

A. That's correct.

Q. So the variable sequence that you talk about in Evans, that's a larger sequence, right?

A. It's -- it's larger than a CDR. A CDR is

a subunit of a variable region.

Q. But smaller than a full antibody?

A. That's correct.

Q. All right.

If we look at Evans in column 19, line 47 -- and, again, we're -- we're -- for the record, we're in AMG-1007, column 19, line 47. You see the sentence beginning "A particularly preferred antibody of the invention"?

A. Yes.

Q. All right. So Evans states "A particularly preferred antibody of the invention is the 5G1.1 antibody (5G1.1, produced by the 5G1.1 hybridoma, ATCC designation HB-11625)"; do you see that?

A. Yes, I do.

Q. So you understand that the -- the phrase "the 5G1.1 antibody" as used here in Evans refers to the original mouse or the murine antibody that's coming out of the 5G1.1 hybridoma, right?

A. Yeah. As we mentioned before, 5G1.1 refers to many things. In this sentence alone it's referring to the hybridoma and the antibody produced by that hybridoma.

Q. Okay. Well, let's -- let's look further

at Evans, then, to column 37. Do you see in example 7 "For preparation of anti-C5 monoclonal antibodies"?

A. Yes, I see that example.

Q. So this example describes the production of the hybridomas reactive to C5 that led to the identification and characterization of the 5G1.1 mouse antibody, right?

A. That's -- that's my understanding of the example, yes.

Q. And if you look at column 38 starting around line 46, you see the second sentence beginning with "Further analyses"; do you see that?

A. Yes, I do see that.

Q. So Evans states "Further analyses reveal that the 5G1.1 antibody produces the cellular" -- sorry -- "produces the cell-lysing ability of complement present in normal human blood so efficiently that even when present at roughly one-half the molar concentration of human C5 in the hemolytic assay it can almost completely neutralize serum hemolytic activity"; do you see that?

A. I do.

Q. All right. And in the next sentence it

states "Immunoblot analysis was undertaken to further" --

THE REPORTER: I'm sorry.

Q. "Immunoblot analysis was undertaken to further characterize the 5G1.1-mAb"; do you see that?

A. Yes, I do.

Q. And in the following paragraph, beginning of the top of page -- column 39 do you see it states that "Assays were performed to determine the relative activity of the N19/8 mAb discussed in examples 4 and 5 with the 5G1.1-mAb in functional hemolytic assays to test and assess whether these mAb's block the cleavage of C5 to yield C5a"?

A. Yes, I see that sentence.

Q. In the next paragraph, column 39 around line 13 you see Evans states that "The 5G1.1-mAb was found to have efficiently inhibited C5a generation at doses identical to those required for the blockade of C5b-mediated hemolytic activity"?

A. Sorry. You lost me. You went from the top paragraph in 39 to somewhere else.

Q. Yeah. No. We're still around line 13.

A. Okay.

Q. You see there's a sentence it begins with

"When the supernatants" --

A. Yes, I do see that.

Q. And if you look at -- so around line 13 it says the -- "The 5G1.1 mAb" -- do you see that -- "was found to have effectively inhibited C5a generation at doses identical to those required for the blockade of C5b activity"?

A. I do see that, yes.

Q. Then there's an example 8 below that in column 39 of Evans; do you see that?

A. Yes.

Q. This -- this example studied the affinity constants --

THE REPORTER: I'm sorry.

Q. -- affinity constants For the anti-human C5 monoclonal antibodies 5G1.1 and N19/8; do you see that?

A. Yes.

Q. And example 9, which starts toward the bottom of column 39 going on to column 40, studied the effect of 5G1.1-mAb on complement activation during CPB; do you see that?

A. Yes, I do.

Q. And then looking at column 40 around line 25, you see a statement saying "These results

further indicate that the 5G1.1-mAb can completely block complement of activation in human blood as measured by either criterion" and it goes on; do you see that?

A. Yes, I do see that.

Q. And you understand that in each of those examples and references that we've just looked at from example 7 through example 9 of Evans the phrase is "the 5G1.1 antibody" or "the 5G1.1-mAb" referred to the mouse 5G1 antibody?

A. That would be my expectation in that context, yes.

Q. Okay. And you agree that the 5G1 mouse antibody described in Evans, examples 7 through 9, that antibody is the only full-length intact 5G1.1 antibody that's described in Evans, right?

A. Again, I'm going to say to the best of the my recollection.

Q. Okay. Example 10 of Evans in column 40 describes the cloning of the variable heavy chain region and variable light chain regions of the mouse 5G1.1 antibody, right?

A. Yes.

Q. And just generally, a POSA prior to March 15th, 2007 would have understood that mouse

antibodies have different amino acid sequences and different clinical properties from -- from humanized antibodies, right?

A. Yes.

Q. Going back to Bell for just a moment. Sitting here today, as far as you're aware, Bell, AMG-1005, doesn't contain any citation to the Bowdish patent application, AMG-1006, right?

A. To the best of my recollection, Bell 2005 does not refer to Bowdish.

Q. And you're not aware sitting here right now of any citations in Bell to Mueller -- I guess either Mueller, either AMG-1008 or AMG-1031, or to Tacken, right?

THE REPORTER: Or what?

MR. DIAMOND: Or to Tacken, AMG-1034.

A. So, again, I don't have complete knowledge of every word and reference in Bell, but to the best of my recollection, you know, those references that you mention are not present within Bell.

Q. And to the best of your recollection, Evans, AMG-1007, also doesn't cite to or incorporate Bowdish or Mueller or Tacken, right?

A. So looking at other publications from the face page of Evans, I don't see those references

listed among those on that page.

Q. And -- and aside from that, from your recollection and your -- whatever studying you did for your deposition and your work on your declarations in this case, you're not aware of any citations in Evans, AMG-1007, to Bowdish or to Mueller or to Tacken, right?

A. I would say that I can't make a specific recollection of references to those publications at this moment.

Q. And you're not aware of any opinions in your declaration that you offered that -- that Evans cites to any of those references, right?

A. I can't recall any such statements within my declaration.

Q. And if it's not in your declaration you're not going to offer that opinion later, right?

A. Yeah. As we talked about, I'm not -- don't expect to have any additional information brought forward at a later time.

Q. Uh-huh.

A. You know, my declaration's my testimony.

Q. Uh-huh.

MR. DIAMOND: This is a good time for a break.

THE VIDEOGRAPHER: We're going off the record. The time is 13:59.

(Whereupon, at 1:59 p.m., the deposition was recessed, to reconvene at 2:45 p.m., this same day.)

THE VIDEOGRAPHER: We're going back on the record. The time is 14:49.

BY MR. DIAMOND:

Q. Dr. Balthasar, at any time earlier today or during the lunch break did you talk to your attorneys about the deposition today?

A. No, I did not. Well, I'm sorry. Not since we started the deposition.

Q. Okay.

Let's talk about -- do we have that? I thought I pulled it out. I guess it's here. Let's talk about Bowdish. The court reporter's going to hand you Bowdish, AMG-1006.

So you have Bowdish, AMG-1006, in front of you?

A. Yes, I do.

Q. And this is the Bowdish reference that you've relied on in several of your anticipation and obviousness grounds in these proceedings, right?

A. Yes, that's correct.

Q. If we look at your '149 Patent declaration, you can go to paragraph 50, and looking at the third sentence you see you have a sentence "The focus of Bowdish"? Do you see that?

A. Yes.

Q. Okay. So a POSA prior to March 15th, 2007 would have understood that the focus of Bowdish is the generation of immunoglobulins or fragments, antibodies, right, that have a peptide of interest inserting into -- inserted into a CDR of an antibody molecule so that the base antibody would serve as a scaffold for presentation of the peptide, right?

A. Yeah. I think that a POSA would understand that Bowdish describes that in part, yep.

Q. Okay. Well, a POSA would understand that's the focus of Bowdish, right? That's what you say in your declaration here, sir?

A. Yeah. I think the way I would think of a term like "focus" or that something focuses on something depends on context. The context of 50 --

Q. Sir, I'm asking you --

A. -- within my declaration --

Q. I'm just asking you a simple question.

A. -- does say that this is the focus of Bowdish.

Q. All right. In your declaration you say that what we just read is the focus of Bowdish,

right?

A. That's correct, and it's correct in the context that it was written. I was just trying to say that there could be other, you know, contexts which you would refer to the focus of Bowdish in a different way, but the way it's referred to within the declaration, paragraph 50 of '149, is exactly the way you said it.

Q. That's the only focus of Bowdish that you identify in paragraph 50, right?

A. That's right. So in that context that's what I'm referring to.

Q. And that's your testimony in your declaration, right?

A. Yep.

Q. Okay.

A. That's my testimony within paragraph 50. So in that context that's exactly right.

Q. Bowdish wasn't about the treatment of complement-mediated conditions such as PNH, right?

A. That's correct.

Q. Bowdish wasn't about the development or study of antibodies directed against C5 or other components of the complement pathway, right?

A. Yeah. I think the -- again, the way that

I would describe the focus is that it's being defined as indicated within paragraph 50 on the "generation of immunoglobulins or fragments thereof that have a peptide of interest inserted into a complementarity determining region, CDR, of an antibody molecule."

Q. There's no mention of C5 or C5 binding anywhere in Bowdish, right, sir?

A. I think that there may be.

Q. Can you point me to where that is?

A. I'll look. So, again, I don't remember the entire document word for word, so it might take me a second to locate it, but one second, please.

(Witness reviewing document.)

A. So my best answer to your question about whether C5 is referred to within Bowdish is that within example No. 4 there's reference of Application Serial No. 08/487,283 and that this is incorporated herein by reference. So that reference has, to my recollection, reference to C5, and my understanding of what that means is that C5 then is referred to in Bowdish.

Q. But in the text on the face of Bowdish itself there's no mention of C5 or C5 binding that you saw, right?

A. So, again, you're asking a specific question. If -- I'm not a lawyer that is very knowledgeable about patent law, but my understanding is that if something is incorporated by reference that it really is part of the text in terms of legal interpretation. If that's correct, then I guess C5 is referred to within the text.

Q. Let's put it that way. Let's put it this way. Apart from the reference that you just referred to to another application, you didn't see any mention of C5 or C5 binding in Bowdish, right?

A. So I think -- I mean, there is incorporation by reference. And with respect to the rest of the document, so I was looking at a particular area for that, I want to spend a little bit more time to answer your question as accurately as possible.

Q. Well, let me just ask you, sitting here right now, today, aside from -- we'll get -- we're going to talk about the incorporation by reference you're referring to.

A. Okay.

Q. But sitting here right now, today, aside -- aside from that document that you say is incorporated by reference, you're not aware of

anything in Bowdish that discusses C5 or C5 binding, right?

A. When you say "discusses," could you be more specific as to what that means? Do you mean specifically indicating C5?

Q. That mentions C5, that provides data regarding C5, that discusses in words C5 in any way. Let me narrow that for you.

Aside from that incorporation by reference, which we'll talk about, you're not aware of anything sitting here right now, today, in the text of Bowdish that discusses C5 binding activity, right?

(Witness reviewing document.)

A. I just want to look to be sure I can answer your question accurately.

Q. How -- in your review of Bowdish sitting here right now, how far along are you?

A. I'm at paragraph 84.

Q. All right. So I'm going to -- for sake of time here, I'm not going to have you reread all of Bowdish, but sitting here today, based upon your knowledge from your preparation for today's deposition, the testimony you're giving today and all the work that you did to prepare for your

declarations in this case from what you recall, you're not aware of anything in Bowdish that discusses C5 binding apart from the incorporation by reference, right?

A. Yeah. I just want to keep reviewing it to be able to answer that.

Q. I'm sure you'd like to spend the rest of your deposition reading this document that you've already offered opinions on in this case, but respectfully, sir, are you familiar with the -- with the main prior art references that you've offered opinions on in this case?

A. Yeah. I think I did, you know, point you to the area where C5 is referred to through incorporation by reference and I think that that demonstrates that I did review this, but I wanted to, you know, just be sure to answer your question accurately. Sorry to offend you, but you asked me a specific question about whether there's reference to C5 within the document. I know there's reference to an antibody that has C5 binding through reference to 5G1.1. So I wanted to be sure that there's no additional C5 mentioned.

Q. My question's different, sir. My question is not asking you to read the whole document.

You've had opportunities -- have you ever read this whole document before cover to cover?

MS. STERLING: Can you watch your tone, please. Lower your voice.

MR. DIAMOND: I'm asking the witness a question.

BY MR. DIAMOND:

Q. Have you ever read the whole document from cover to cover, sir?

A. No, I've not read the whole document from cover to cover.

Q. Okay. Well, we're not going to have you do that for the first time now, but sitting here today, based upon what you've reviewed to date in Bowdish AMG-1006, you're aware of incorporation by reference, we'll talk about that, but aside from that, you're not aware of anything else sitting here right now, this minute in Bowdish that you could point me to where they talk about C5 binding, right?

A. Yeah. So I don't have a perfect recollection, a photographic memory to be able to --

Q. I'm not asking you for that, sir.

A. No. You said --

Q. I'm asking you what you recall right now.
A. You asked me, though, of what I've reviewed in the past in preparing for this.
Q. Let's make it a real simple question, then. Sitting here today, based upon what you recall right now in your chair this minute, you can't point me to anything else in Bowdish that you say references C5 binding aside from that one statement regarding incorporation by reference, right?
A. There is that paragraph that refers to incorporation by reference that relates I think by reference -- you know, incorporation of that reference to C5 binding, and I'm not able at this moment to point to any other reference.
Q. Fair enough.
Why don't you turn to paragraph -- actually, before we do that, you agree that Bowdish doesn't disclose the entire amino acid sequence of Sequence ID No. 2 and Sequence ID No. 4 that's recited in the claims of the challenged patents, right?
A. Yeah, that's correct. As I've indicated in the declaration, it discloses the entire sequence except for CDRH3 for eculizumab.

Q. So it doesn't disclose the entire claimed sequence, right?

A. That's correct. It discloses everything except CDRH3 for eculizumab.

Q. And the amino acid sequences that you rely on from Bowdish are the sequences disclosed in figures 13A and 13B, which appear on pages, using the bottom page number, 17 and 18 of the document, right?

A. Yes, that's correct.

Q. And you agree that the antibody of Bowdish with the amino acid sequences in figures 13A and 13B, that -- that antibody is not the antibody claimed in the challenged patents, right?

A. I don't know that I would describe the sequence of 13A and 13B as being consistent with an antibody. It's more of a fusion protein.

Q. Okay. Let me rephrase it, then. Is it okay if we call it a construct?

A. Sure.

Q. All right. So that -- so that construct or fusion protein represented by the sequences 13A and 13B in Bowdish, that's not the claimed antibody of the challenged patents, right?

MS. STERLING: Objection to form.

A. It's correct. The sequence that's given in 13A and 13B are the sequences for the claimed antibodies absent the CDR heavy chain 3 domain.

Q. Okay.

A. Which is replaced with a TPO mimetic.

Q. There's nothing in Bowdish that says that the -- that the fusion protein or construct made of figures 13A and 13B would bind C5, right?

A. I think the incorporation by reference, to my understanding of the legal understanding of that, would give description of C5 antibodies --

Q. Sir, focus on my question here. My question is about the sequences in figures 13A and 13B.

A. Uh-huh.

Q. Okay? Are you with me?

A. Uh-huh.

Q. So there's nothing in Bowdish that says that a fusion protein or construct comprised of the sequenced cited in figures 13A and 13B of Bowdish would bind C5, right?

A. So, again, I think it's a complex question. My understanding is that Bowdish incorporates by reference Evans, Evans refers to the CDR regions for the 5G1.1 antibody that do bind

to C5.

Q. Is the construct comprised of figures 13A and 13B of Bowdish, is that entire sequence disclosed in Evans? No, right?

A. So the entire sequence for the TPO mimetic agent is not disclosed within Evans, but Evans does disclose CDR domains. Those CDR domains, to my understanding, include domains that would interact with C5.

Q. This entire sequence in figures 13A and 13B, I want you to take the sequence in its entirety, it's not disclosed in Evans, right? The sequence -- not the CDR's, the sequence in its entirety is not disclosed in its entirety in Evans, right?

A. That's correct.

Q. Okay. So is there anything in Bowdish that says that the fusion protein made of the sequences in figures 13A and 13B as they are, that that fusion protein would bind C5?

A. So a POSA would have the expectation that the sequence that is present in 13A and 13B may very well bind C5 through the knowledge that's given in the incorporation by reference of Evans because the binding domains are the CDR regions

that interact with C5. Those are present within the sequence excepting CDRH3.

So that's the link, that because those binding domains are part of the sequence, a POSA would think it's possible, if not likely, that this sequence would bind to C5.

Q. I'm not asking you for your opinion right now about what you think a POSA might say is likely or possible. I'm asking you whether there's anything in Bowdish -- and if there is, please point me to where it is -- that says, supported by data, right, that this sequence in figures 13A and 13B, that that fusion protein would bind C5?

A. I'm doing the best I can to answer your question. So my --

Q. Let me ask you a different question, then.

A. My --

Q. Let me ask you a different question.

A. My answer is that the CDR regions --

Q. I'm going to --

A. -- which are present --

Q. Sir, I'm withdrawing that question. Don't -- don't -- I know you're not going to answer it.

My question is do you know sitting here

today one way or the other under oath that the fusion protein represented by figures 13A and 13B does or doesn't bind C5?

A. I would say that I do not have a, you know, experiment that I have conducted that makes use of an antibody with this sequence where I've demonstrated C5 binding --

Q. So under oath can you say --

A. but --

Q. -- can you say that you know --

A. -- but for the Evans reference, which discloses the CDR regions that are incorporated within this sequence, it is within that publication that the antibody related to those binding domains does bind C5. So I have a difficult time to be able to, I think, give you what you want in that there's knowledge that the CDR regions, the binding domains bind to C5.

Q. Sir, what I want -- what I want is you to answer my question. You understand you're under oath today, right?

A. Yes.

Q. Okay. You understand you're testifying under penalty of perjury today, right?

A. Correct.

Q. Okay. So can you under oath today tell me with a hundred percent certainty that the antibody or fusion protein represented by figures 13A and 13B recited in Bowdish does bind to C5? Can you tell me that, sir? And if you can't tell me that with a hundred percent certainty, then that's your answer. Please say it.

A. So I don't know that there's much of anything relating to antibody sequence or activity that I could say with a hundred percent certainty, but what I can say --

Q. Well, let me -- let me --

A. -- is that the CDR regions --

Q. Yeah?

A. -- that are present within the sequence are identified within Evans --

Q. Sir --

A. -- as binding to C5.

Q. -- you're not answering my question. Are you aware of any data actually studying the C5 binding of this fusion protein represented by figures 13A and 13B in Bowdish, yes or no? Are you aware of any such data?

A. So I'm aware of data that relates to fragments --

Q. You're answering a different question, sir.

A. Fragments that --

Q. Are you aware of any binding data with respect to this complete construct represented by figures 13A and 13B, yes or no, sir?

A. For this -- for a protein that is of this complete sequence, I have not seen or have personal knowledge of any information that relates to binding.

Q. Okay. Thank you.

So let's go to the paragraph in Bowdish you want to focus on, paragraph 191. So this is the paragraph that contains the statement regarding the incorporation by reference that you were talking about earlier?

A. Yes.

Q. And let's actually look at your declaration, your '149 declaration, paragraph 94.

A. Sorry. Paragraph 94?

Q. Yeah.

So I'm referring you specifically to the last sentence in that paragraph which begins on page 61 towards the top of the page that says "In this case." Let me know when you see that

sentence.

A. Yes, I see that sentence.

Q. So you say here that "In this case because Bowdish incorporates Evans by reference for the construction of 5G1.1, it is as if the teachings in Evans for construction of 5G1.1 are also recited within Bowdish." You see that in your declaration, right?

A. Yes. That's my understanding.

Q. Okay. So let's look back at Bowdish. I'm sorry. That's your understanding of the -- of how Bowdish incorporates Evans by reference?

A. No. That's my understanding with respect to the teachings of Evans for construction of 5G1.1 were also recited within Bowdish. I was saying that that's my understanding about what the meaning of this sentence is.

Q. Okay. The meaning of this sentence in your?

A. Declaration.

Q. Okay. So in your -- in your declaration -- let me rephrase that.

It's your understanding that because Bowdish incorporates Evans by reference for the construction of 5G1.1, it's your understanding that

it's as if the teachings in Evans regarding construction of 5G1.1 were also recited within Bowdish? That's your understanding of how this operates?

A. So this sentence, you know, reads in a way that's consistent with what you said, but my understanding of incorporation by reference extends beyond that. My understanding of incorporation by reference is that all of the teachings of the reference are as though they're recited within Bowdish.

Q. Who told you that?

A. That's my understanding through discussion with counsel.

Q. Okay. And you're not an expert in the law on that issue, right?

A. Correct.

Q. And, of course, with respect to that legal understanding and the other legal understandings you've received from counsel, you've used those as a basis for forming your opinions in this case, right?

A. Yes. My understandings that I've gained through counsel with respect to legal matters are part of the basis for opinions that I've drawn

within the declaration.

Q. Okay. Let's take a look at paragraph 191 of Bowdish. You see it says in the second sentence, it begins "Construction of 5G1.1"; do you see that?

A. Yes, I do see that.

Q. It says "Construction of 5G1.1 is described in the U.S. Application Serial No. 08/487,283, incorporated by -- herein by reference"; do you see that?

A. Yes, I do.

Q. Now, you understand that the document referenced here, U.S. Application Serial No. 08/487,283, you understand that that's not the Evans patent that you have in front of you, AMG-1007, right?

A. Yeah. My understanding, as indicated in paragraph 94 of my declaration '149, is that U.S. Application No. 08/487,283 issued as Evans, which we reference as AMG-1007.

Q. Right. So you understand there was an application with a number ending in 283 and that application at some point issued as the Evans patent that is AMG-1007, right?

A. That's my understanding.

Q. And if we refer to this application as in contrast to the Evans patent, if I refer to this application referenced in paragraph 191 of Bowdish as the '283 application, will you understand what I'm saying?

A. Yes, I will.

Q. The '283 application isn't one of the documents that you listed in your tables of the documents you considered for purposes of forming your opinions in this case, right?

A. That's correct. My understanding is that that document issued as Evans, which is document No. AMG-1007.

Q. You don't cite to the '283 application by reference to any like AMG exhibit number the way that you cite to the other exhibits addressed in your declarations, right?

A. Yeah, that's right. Because it's issued as Evans, I cite to Evans AMG-1007.

Q. Let's look at paragraph 114 of your '149 Patent declaration, and let me know when you're there, sir.

A. I'm there.

Q. All right. Here you're discussing your position that a POSA would have had a reason to

combine the Bell, Bowdish, and Evans references to arrive at the antibody recited in claim 1 of the '149 Patent?

A. That's correct. That's the heading of the section, yes.

Q. That's the general content of this section, right?

A. Yes.

Q. Okay. And in support of your ground 4 for the '149 Patent -- let me rephrase that.

This section here is in support of your ground 4 for the '149 Patent, which is your assertion that claim 1 of the '149 Patent would have been obvious in view of the Bell, Bowdish, and Evans references, right?

A. Yeah, that's right. It's a component of this claim for -- this argument for obviousness, yes.

Q. Okay. You understand that in order to establish obviousness of a claimed invention in view of a combination of prior art that the reason to combine the prior art references must be shown, right?

A. Yes. I understand that there must be a reason to combine those references as part of the

obviousness argument, yes.

Q. And you also understand that as part of the obviousness argument there must be a reasonable expectation of success that that claimed combination would work for its intended purpose, right?

A. Yes, that's my understanding.

Q. So looking at paragraph 114 of your '149 declaration, in your contention that claim 1 of the '149 Patent is obvious in view of Bell, Bowdish, and Evans you argue that Bell, AMG-1005, would have motivated the POSA to make eculizumab which Bell described as an anti-C5 antibody that successfully treated PNH patients, right?

A. Yes. That's the gist of the first sentence, yes.

Q. And you understand that Bell didn't provide the amino acid sequence of eculizumab, right?

A. Yes, that's my understanding.

Q. So you say that a POSA would have had reason to look to other references to find the sequence of eculizumab, right?

A. Yes, that's correct.

Q. As we discussed earlier, the paragraph of

Bell that talks about methods of making eculizumab and various humanized fragments and scFv's, that cites to the Thomas and Evans references, right?

A. You know, it's possible. I'd probably want to look at that again to be able to, yeah.

Q. Okay. I think as you said earlier, you weren't aware of anything in Bell that cites to Bowdish, right?

A. I can't remember exactly what I said earlier, but I'm sure it's in the record if you want to search for it to read it back.

Q. I'll ask you again because it should be a simple one. But you're not aware sitting here today of anything in Bell that cites to Bowdish, right?

A. I'm sorry. I thought you were asking if I had testified to that earlier or mentioned that earlier.

Q. I thought you did, but it's such an easy question I thought I'd ask you again.

A. So again the question?

Q. You're not aware of anything in Bell that cites to Bowdish, are you?

A. Let's see.

(Witness reviewing document.)

A. Again, I don't have hundred percent recollection of every word or phrase used within Bell, but my recollection is that there's no citation to Bowdish.

Q. All right. If you could go to paragraph 115 of your '149 declaration, you can look further there. Here you provide your reasons why you say a POSA would have combined Bell's teachings regarding eculizumab with the sequences in Bowdish, right?

A. Yes, that's -- so paragraph 115 --

Q. Right.

A. -- refers to the reason to look to Bowdish.

Q. Okay. And the reasons you offer here for combining Bell and Bowdish are that -- you say Bowdish provides most of the sequence of humanized h5G1.1 with a hybrid IgG2/G4 constant region as the framework of its 5G1.1 TPO antibody, and you say eculizumab, which is the antibody described in Bell, was also known as a humanized h5G1.1 with a hybrid IgG2/IgG4 constant region, right?

A. Yes, that's correct.

Q. So in your reasoning here, for a POSA to have been motivated to combine Bell and Bowdish,

they would have had to know that Bowdish's antibody was a humanized antibody with an IgG2/IgG4 constant region and also that eculizumab had an IgG2/IgG4 heavy chain constant region, right?

A. So I'm sorry. That was a long question. Can you restate it?

Q. Sure. I'm just trying to understand the reasoning here in your paragraph 115, and for a POSA to have been -- in your argument -- I'm just taking your argument here. In your argument in paragraph 115 for a POSA to have been motivated to combine Bell and Bowdish the POSA would have had to know that Bowdish's antibody was a humanized antibody with an IgG2/IgG4 constant region, right?

A. So I think for -- the reason to look to Bowdish -- if I was a POSA interested in developing eculizumab, the motivation to get to Bowdish would be that eculizumab is known as a humanized G1, I would search the literature for sources that give information about humanized 5G1.1 antibodies and in that search I would find Bowdish. But to go forward with the combination --

Q. I think you might have misspoke. You said humanized G1?

A. I meant 5G1.1, yes.

Q. Okay. So -- well, that's not what you say -- let's look at what you say in your paragraph 115. Okay?

A. Sure.

Q. You say "A POSA would have had reason to look at Bowdish because it provides most of the sequence of humanized 5G1.1 with a hybrid IgG2/G4 constant region as the framework of its 5G1.1-TPO antibody," right?

A. Yes.

Q. And you say "A POSA would have linked this disclosure to Bell because eculizumab was also known" -- it says "also known to a humanized 5G1.1 with a hybrid IgG2/IgG4 constant region"; do you see that?

A. Yeah. I think that, you know, is a typo between "to" and "a." Probably "known to be a" --

Q. "Known to be"?

A. Yeah.

Q. Yeah. I was going to ask you that. Okay. So let's -- so what you're saying here in paragraph 115 is that a POSA would have associated their knowledge that Bowdish disclosed a humanized 5G1.1 with a hybrid IgG2/IgG4 constant region with their knowledge that eculizumab was a humanized

5G1.1 with a hybrid IgG2/IgG4 constant region, right?

A. Yeah. I think that's pretty close to exactly what's written there. I mean, the exact words are "And a POSA would have linked this disclosure to Bell because eculizumab was known to be a humanized 5G1.1 with a hybrid IgG2/IgG4 constant region."

Q. There's no mention of eculizumab in Bowdish, right?

A. Bowdish does not make direct use of the term "eculizumab."

Q. Okay.

A. That I recall.

Q. Neither does Evans, right?

A. Yeah. Evans, to my recollection, does not make use of the direct term "eculizumab."

Q. Okay.

Bowdish doesn't expressly say that the scaffold at the body that was used to generate the 5G1.1-TPO fusion protein was a humanized antibody, right?

A. That's correct.

Q. And Bowdish doesn't use the phrase "h5G1.1," right?

A. Let me look back at Bowdish.

Q. If you want to take a look and just to focus you, I think we can go back to example 4 of Bowdish starting in paragraph 191. I'll ask you that question again, but before we do that, paragraphs -- the section of Bowdish that you focus on for purposes of this case is what's disclosed in paragraphs 191 to 193 in example 4 as well as the figures that are referenced in that paragraph, right? Or in those paragraphs.

Would you agree that example 4 of Bowdish and the figures that it references is what you've been focusing on with respect to Bowdish's disclosures for purposes of these proceedings?

A. Yes. It's correct that I've looked at Bowdish with respect to the sequence that is related to example 4 and shown in the figures 13A and 13B, and my opinion of how one gets there is by linkage to 5G1.1, which is kind of a synonym for eculizumab as we've talked about before, and the sequence itself gives information about the nature of the sequence, including that it's human and derived of human G2 and G4 constant domains.

Q. The portions of Bowdish that you've focused on for purposes of this proceeding don't

use the term "h5G1.1," right?

A. Right. They use just 5G1.1 --

Q. Okay.

A. -- which is --

Q. Okay.

A. -- recognized as a synonym for eculizumab in some citations.

Q. Okay. You cite -- we're going to hand you a document. This is AMG-1021. You've mentioned a few times that the term 5G1.1 without a leading H has been used in the part sometimes to refer to eculizumab, right?

A. That's right, and this reference is an example of that. It's also used to refer to other things as well as we've talked about previously today.

Q. Okay. Fair enough. So this reference we're looking at is the Kaplan reference, which is AMG-1021, right?

A. Yes. I recognize this.

Q. Okay. And the title is "Eculizumab Alexion"; do you see that?

A. Yes.

Q. And I guess what you're relying on is what's in the left-hand column where it says

"Eculizumab" and then "(5G1.1), a humanized monoclonal antibody" and goes on?

A. That's one place within the reference that eculizumab is referred to as 5G1.1. Within the lead-in portion, I guess part of the abstract, the first sentence starts with "Eculizumab (5G1.1)" and then goes on "A humanized monoclonal antibody" from there.

Q. Okay. And on the right-hand side there are synonyms listed, right?

A. That's correct.

Q. But the synonyms aren't quite correct because h5G1.1 isn't necessarily a synonym for h5G1.1-scFv, right?

A. So I'm sorry. Could you repeat that? H5G1.1 you're saying --

Q. H5G1.1 is a different construct than h5G1.1-scFv, right?

A. Full-length 5G1.1-mAb is a different construct than the 5G1.1-scFv, that's correct.

Q. So when -- in the right-hand box when it's saying 5G1.1 is a synonym for these other things, what do you take that to mean?

A. As we talked about earlier today, in the development of antibodies that starts usually from

a well within a microplate that is numbered based on the plate, the row, the column associated with that well. And so in the case of the anti-C5 antibodies we've been talking about, that well, to my understanding, was 5G1.1, and that name is used to refer to many things.

It refers to not only the well, but the hybridoma cells within the well, the antibody produced by those hybridoma cells, and often is used to describe variants of the antibody, including in some cases humanized antibodies. In some cases that same label is used to talk about chimeric antibodies. In some cases that same label is used to talk about fragments.

There's many examples of that throughout the literature. So, you know, in some contexts they're saying basically this is used interchangeably in different ways.

Q. Fair enough.

Let's go to page 2 -- let's go to page 1018 of Kaplan and go to the left-hand side. You see a sentence beginning -- or paragraph beginning "Several complement inhibitors"; do you see that?

A. I do see that.

Q. The next sentence reads "Eculizumab (5G1.1) under development by Alexion Pharmaceuticals is a humanized C5 inhibitory monoclonal antibody (mAb) that prevents cleavage of the human complement component C5" and it goes on?

A. I do see that, yeah.

Q. All right. So in the context of this Kaplan paper they're using 5G1.1 to refer to eculizumab, right?

A. Yeah. In the context of this paper that's correct. That's my understanding.

Q. Fair enough.

Then if you look in the right-hand column there's a -- that same page, there's a discussion of synthesis and SAR, right?

A. Yes, I do see that.

Q. And they talk about how humanized 5G1.1-FaB and scFv molecules were produced by grafting the complementary -- complement- -- sorry -- grafting the complementary determining regions of 5G1.1 onto human framework regions; do you see that?

A. I do. I know that you kind of repeated complementary a couple times. That's obviously a typo as well.

Q. Right. That's why I got tripped up there, but you understand it to be referring to the CDR's, right?

A. Yes.

Q. Okay. And there's a reference 258884 that's cited for that sentence; do you see that?

A. Yes, I do see that.

Q. And then if you go further down, you see a sentence -- in that same paragraph you see a sentence that says "In addition"; do you see that?

A. Let me see.

Q. Just let me know -- I'll go beyond that, but I want you to let me know when you see the "In addition, intact humanized." Do you see that sentence? It's about a little bit of a way --

A. Yes, I do see that.

Q. Okay. So that sentence in the right column of page 1018 of Kaplan says "In addition, intact humanized 5G1.1 antibody blocked human complement lytic activity at concentrations identical to the original murine monoclonal antibody indicating that humanized 5G1.1 and its derivatives retained both the affinity and blocking functions of murine 5G1.1 antibody"; do you see that?

A. I do see that.

Q. And it cites two references there, 258883 and 258884, for that statement, right?

A. Yes, I do see that.

Q. It goes on to say that "5G1.1 is supplied as a sterile, nonpyrogenic solution (2 milligrams per milliliter) for IV injection" and cites two references, 212467 and 258884; do you see that?

A. Yes, I do see that.

Q. And reference -- if you go to page 1022 of Kaplan and look at the bottom left-hand corner, do you see that reference 258884 is Thomas, AMG-1023, right?

A. Yes, I see that.

Q. Okay.

A. 25884 -- I'm sorry -- 8884 is referring to the Thomas paper that we talked about earlier.

Q. Okay. You can put that down.

Just going back to Bowdish for a second, and I want to refer you back to paragraph 191 that we were looking at.

A. I just turned the page. Okay. I'm back.

Q. Back to the statement that says "Construction of 5G1.1" that we were looking at earlier. Do you have that?

A. Yes.

Q. All right. Do you see that the document that was incorporated by reference here was the '283 application, not the Evans patent itself, right?

A. Yeah. My understanding is the '283 application issued as Evans, yes.

Q. My question is simply you understand that the document incorporated by reference here in paragraph 191 is the '283 application, not the Evans patent itself, right?

A. And I was just trying to clarify that my understanding is that this document, the '283 application, issued as Evans.

Q. That clarification's not answering my question. Let's take it one piece at a time.

I understand -- I understand -- let me rephrase that.

The document referenced in paragraph 191 is the '283 application, right?

A. I see that reference.

Q. You don't see the patent number listed for the Evans patent here, right?

A. Yeah. I don't see the listing of the patent number that we've been referring to as

Evans, but I do know, as we've said, that the '283 application issues as Evans.

Q. You don't know whether or not the '283 application was published, right?

A. It's not an application that I looked at in any type of form to consider for the declaration. It's not one of the documents that we've cited that you mentioned earlier.

Q. Now, you said, I believe earlier, that you think a POSA reading Bowdish would have somehow determined or inferred that Bowdish's sequences had an IgG2/IgG4 constant region, right?

A. Yeah. I would think that not necessarily by reading it, but you would analyze the sequence by using a tool like Blast and then make that determination that these are human sequences that relate to human G2 and G4. You would, you know, find that through that type of tool.

Q. And you also offer an opinion in this case that the POSA might -- you say a POSA might think that the antibody was humanized because of the goat anti-human region -- goat anti-human reagent used in the assay described in example 4?

A. That's correct. That's in the declarations.

Q. Let's talk about those issues. First, why don't we talk about the 5G1 -- sorry -- the antibody reagent.

A. Could you direct me to where it is in the declaration just to refresh my memory?

Q. I think you talk about it in paragraph 90, for example, your '149 declaration.

A. Thank you.

Q. And you say that "A POSA would have understood that the 5G1.1 antibody used in Bowdish is a humanized 5G1.1 antibody because Bowdish used an anti-human antibody to detect the presence of 5G1.1," right?

A. Sorry. I turned to the paragraph in the wrong document.

Q. No worries. It's paragraph 90 of your '149 declaration.

A. Yes, I'm there.

Q. All right. So, again, you state -- you state that "A POSA would have understood that the 5G1.1 antibody used in Bowdish is a humanized 5G1.1 antibody because Bowdish used an anti-human antibody to detect the presence of 5G1.1," right?

A. Yes, that's right.

Q. So that's something that you're inferring

based upon Bowdish's use of that antibody, right? As we said, Bowdish doesn't mention that it's humanized, right?

A. Yeah. It's just an expectation that a POSA would -- would make is what I'm trying to state here.

Q. You cite to paragraph 192 of Bowdish for that proposition regarding the reagent, right?

A. That's right.

Q. And if you turn to paragraph 192 of Bowdish, and let me know when you're there.

A. I'm there.

Q. Bowdish doesn't say what the source was of that assay reagent, right?

A. Yeah. I don't see reference within this paragraph and I don't recall reference throughout Bowdish for the source of the reagent.

Q. Sitting here today, you're not aware of any information in Bowdish regarding the specific epitope for that reagent, right?

A. No. In fact, with these preparations most commonly they're polyclonal. So it's -- yeah, this definitely is. It's goat anti-human IgG. The H and L in parentheses is indicating a (indecipherable) against heavy and light chains.

So --

THE REPORTER: I'm sorry.

A. I'm sorry. So what is referred to is a preparation that is a goat anti-human IgG preparation. It has in parentheses H and L, and what a POSA would understand that to mean is that this is referring to the preparation interacting with heavy and light chains of the antibody, meaning that multiple epitopes are being bound by the anti-human IgG preparation indicating it's not a monoclonal, but would be a polyclonal preparation.

Q. It doesn't specify whether that anti-human antibody reagent, whether it detects both humanized and chimeric antibodies, right?

A. Yeah. It doesn't make that description. So you're correct, I don't see any further information about the reagent listed.

Q. And you don't see any information about the cross-reactivity of that reagent with, for example, mouse IgG antibodies or antibodies of other species, right?

A. Yeah. I just see definition and then it says anti-human, and the reason why I would say that there's -- you know, I don't have concern

about a chimeric is that a chimeric antibody, of course, would be typically human -- human chimeric of a mouse. So it has many human epitopes with that. So it's a form of a humanized antibody.

Q. Okay. So an anti-human antibody could potentially bind to both chimeric antibodies with a mouse variable region, but a human Fc as well as humanized antibody?

A. That's correct. And --

Q. You -- let me -- I have a different question. Sorry.

Bowdish doesn't say anywhere -- I think we established Bowdish doesn't say anywhere that the antibody constructs that it used are -- have a hybrid IgG2/IgG4 region, right?

A. The only information that's relevant to that, to my knowledge, is the sequence, which the sequence, you know, when analyzed clearly indicates that it's G2/G4 constant domains present.

Q. In your -- in your declaration in this case -- and I think you just said it too, but I think in your declaration in this case you say that a POSA would have used some kind of search tool or could have used some kind of search tool to determine that sequence being, as you say, a

humanized IgG2/IgG4; is that right?

A. Yeah. A search tool like Blast would have identified regions of the sequence that are human IgG2 constant domains and IgG4 constant domains and would have no question then that this is an antibody that is containing these human regions, yes.

Q. In your -- so that's not information, I guess, regarding -- let me rephrase that.

What the identity is of the constant regions in the constructs described in example 4 of Bowdish, that's not information that's expressly described in Bowdish. That's something that a POSA, you say, could determine by taking information that's in Bowdish and then putting it into some kind of search tool, right?

MS. STERLING: Objection, form.

A. Yeah. I mean, I think that the reference in the paragraph that we went through about the detection antibody being an anti-human antibody, you know, gives some indication that it's human, that the construct is human, but -- or reacts with its human detecting antibody, but the information about the sequence is not directly stating anywhere that it's human. It's something that you would

determine when you analyze the sequence.

Q. Right. So it's not something that's stated directly in Bowdish. It's something that you'd have to take the sequence and then put it into a search tool and the search tool would give you information, right?

A. That's correct. I mean, it's a little bit of semantics. The sequence is defining -- you know, the sequence defines the antibodies that it comes from or that it relates to.

Q. Okay.

A. So it's a component of the sequence I guess.

Q. Let me ask you this. Well, there's no search tool actually in Bowdish, right?

A. That's correct.

Q. Okay. For purposes of this case in your let me expressly -- let me rephrase.

In your declarations here in this case you don't point to anything in Bowdish that instructed a POSA to use Blast or a similar search tool, right?

A. I don't make any statement like that in the declaration, right.

Q. Okay. And in your declarations in these

proceedings you didn't provide any actual demonstration of the search results that a POSA would have gotten if prior to March 15th, 2007 they had tried to evaluate the sequences in Bowdish figures 13A and 13B using Blast or a similar search tool, right?

A. That's correct. I didn't include any demonstration.

Q. Okay. Let's go to --

A. I guess I would say -- sorry. Maybe I'm -- I didn't include any demonstration of an application of Blast in the sequence, but there is -- you could tie the sequence to Bowdish --

Q. Sir --

A. -- to Sequence ID 2 and 4.

Q. Sir, I'm not asking you -- I'm not asking you -- I'm asking you what you did in your declarations here, and you didn't do that, right?

A. I didn't include any demonstration of a Blast analysis of the sequence, yeah.

Q. Okay. Or analysis using any other search tool, right?

A. Yeah, with any other search tool, but there was, I guess, analysis that one would point to --

Q. Thank you. Thank you. Thank you.

A. Yeah.

Q. Let's look at paragraph 149 of your declaration -- I'm sorry -- 118 of your '149 declaration. You say here that "A POSA" -- are you there?

A. Yes, sir.

Q. You say here that a POSA would have had a reasonable expectation of success in performing the molecular biology techniques to combine sequences from Bowdish and Evans, right?

MS. STERLING: Objection, form.

A. Let's see. I think the statement is "A POSA would have had a reasonable expectation of success in making the antibody of claim 1 from the disclosures of Bell, Bowdish, and Evans because doing so would have only required basic molecular and cellular biology techniques." That's the statement.

Q. Right. What you're saying here is that a POSA would have had a reasonable expectation of success in making an antibody with the sequences recited in claim 1 using basic molecular and cellular biology techniques, right?

A. That's close to exactly what's there.

It's just making the antibody of claim 1 from disclosures of Bell, Bowdish, and Evans because doing so would have only required only basic molecular and cellular biology techniques.

Q. In this paragraph you don't say why a person of skill in the art -- I'm focusing you on -- are you with me in paragraph 118 of your declaration, sir?

A. Yeah. I'm trying to listen to your question.

Q. Yeah. So in this paragraph here regarding reasonable expectation of success you don't say why a POSA would have had a reasonable expectation that the resulting antibody from combining sequences of Bowdish and Evans would be an antibody that binds C5? You don't say that here, right?

A. So sorry. Could you please repeat that question. It was a long question.

Q. In your paragraph 118 regarding reasonable expectation of success --

A. Uh-huh.

Q. -- you don't address why a POSA would have had a reasonable expectation that the antibody resulting from combining sequences of Bowdish and Evans would be an antibody that binds C5, right?

A. So within this section what is discussed is the reasonable expectation of success that you would generate a humanized 5G1.1 antibody --

Q. So, sir, do you hear --

A. -- with a G2/G4 --

Q. -- do you hear my question?

A. -- domain --

Q. My question's different.

A. -- which is eculizumab --

Q. Sir. Sir. Sir.

A. -- which is known to bind C5.

Q. Sir, sir, I'm focusing you on your declaration here, what's in this paragraph. Okay? In this paragraph -- you don't mention C5 binding in this paragraph, right?

A. Yeah, but it's referred to with respect to claim 1.

Q. Sir, you don't mention C5 binding in this paragraph, right?

A. I don't expressly mention C5 binding --

Q. Thank you.

A. -- in the paragraph --

Q. Thank you.

A. -- but it's --

Q. Thank you. No.

A. -- what --

Q. That's my question, sir.

A. -- claim 1 is related to.

Q. That's my -- that's my question. I'm not asking you that other question you're asking yourself, sir. You have to stick with my questions. Okay?

And in your declarations in this case you don't point to any C5 binding data from any reference showing that the construct you combine from sequences in Bowdish and Evans binds C5, right?

A. Your question is very complex. Are you asking me if I've included in the declaration a demonstration of C5 binding?

Q. I'm asking you if you included anything in your declaration from the prior art, so it's got to be prior to March 15, 2007, containing data showing that the antibody that you derive from combining sequences from Bowdish and Evans in your declaration binds C5?

A. So, again, it's a complex question, but the way I can answer it to the best of my ability is that the information that is cited with respect to Evans, which is discussing humanized versions of

the antibody 5G1.1, refers to sequence for CDR regions, and CDR regions are the antibody binding domains that are known to interact with C5. And by referring to antibodies incorporating sequence relating to those CDR's, the function follows the sequence, the sequence dictates that you would have binding to C5. That's my understanding.

Q. Sir, that's not my question. My question is did you include anything in your declarations from the prior art containing actual data, not assumptions, actual data in the binding study showing that the antibody that you derive from combining sequences in Bowdish and Evans binds C5?

A. So the antibody that is associated with the sequence that relates to the combination --

Q. Sir, sir, sir.

A. I have -- it's a long question that you've given me.

Q. It's a simple question.

A. I have to explain it.

Q. Do you cite that data, yes or no?

A. So the sequence that is the combination of Bowdish and Evans is the sequence of Sequence ID No. 2 and No. 4 which relates to eculizumab which is discussed throughout the declaration as an

anti-C5 antibody. So in that context there's a relationship between sequence binding to C5 and eculizumab, and that's what I'm really talking about.

Q. So you're relying on the references that talk about the C5 binding properties of eculizumab?

MS. STERLING: Objection, misstates the witness's testimony.

A. So the relationship that I'm relying on relates -- the sequences of binding domains of G51.1 [sic] antibodies to binding to C5 in activity, you know, in some cases of those antibodies, that is information provided by Evans.

Q. But Evans doesn't disclose the full antibody?

A. No. It just discloses in part the antibody binding domains called the CDR regions or complementary --

Q. Okay.

A. -- determining regions --

Q. Okay.

A. -- the regions that are responsible for binding.

MR. DIAMOND: Okay. This is a good time for a break.

THE VIDEOGRAPHER: We're going off the record. The time is 15:59.

(A short break was had.)

THE VIDEOGRAPHER: We're going back on the record. The time is 16:17.

BY MR. DIAMOND:

Q. Dr. Balthasar, in your three declarations you have a number of figures where you compare the sequences of Sequence No. 2 and Sequence No. 4 that are claimed in the challenged patents with the various sequences that you take from the prior art that you rely on, right?

A. That's correct.

Q. For example, if you look at your '149 declaration and go to page 35.

A. Yes.

Q. And here you're comparing Bowdish's sequences from figures 13A and 13B to Sequence ID Nos. 2 and 4 of the antibody claimed in the challenged patents, right?

A. That's correct.

Q. And if we go to page 37, you have figures 6 and 7 there, right?

A. Yes, that's correct.

Q. Here you're comparing figure -- Sequence

ID No. 20, which is an scFv from Evans, to the light and heavy chain variable regions of the sequences recited and claimed in the challenged patents, right?

A. Yeah, that's right.

Q. Okay. And you understand that Evans disclosed at least nine exemplary humanized scFv sequences, right?

A. That's correct.

Q. Okay. In paragraph 56, so this is on page 40 of your '149 declaration, you're comparing Mueller's 3F4 heavy and light chain constant regions to the heavy and light chain constant regions of Sequence ID No. 2 and, if you go onto the next page, Sequence ID No. 4, again, taken from the '149 Patent, right?

A. That's right.

Q. If you go to figure No. 13, which is in paragraph 99 of the '149 declaration, this is on page 64, here you're comparing the sequence that you get from combining sequences from Bowdish and from Evans with Sequence ID No. 2 of the '149 Patent, right?

A. That's correct.

Q. When you generate -- well, did you

generate these figures?

A. No. Counsel prepared the figures.

Q. Okay. You understand that a person of skill in the art prior to March 15th, 2007 would not have had the '149 Patent, right?

A. So the '149 Patent issued after 2007, that's correct.

Q. And it was filed after that date, right? It was filed on March 15th, 2007 and it issued after that date, right?

A. That's correct. Well, or maybe is it the 16th? The 15th. Sorry. Yeah, that's right.

Q. Likewise, the '880 Patent and the '504 Patent, you understand that a person of ordinary skill in the art prior to March 15th, 2007 wouldn't have had access to those documents either, right?

A. Correct.

Q. So a person of skill in the art prior to March 15th, 2007 could not have done these sequence comparisons between the prior art literature and the sequences disclosed in the challenged patents, right?

A. Yes, that's correct. The challenged patents were, you know, publicly available at a

later date.

Q. Okay.

Let's turn to your figure 12 in the '149 Patent. Who generated this figure?

A. I'm sorry. Could you direct me to the page?

Q. It's on page 61 and 62 --

A. Okay. So yeah. So the figure --

Q. -- of the '149 declaration.

A. -- on page 62 was generated with counsel through discussion. So counsel actually did the -- you know, the processing of the figure.

Q. Uh-huh.

A. We talked about, you know, how to create the figure and this is what we came up with.

Q. Okay. And you say that you generated -- this figure was generated to illustrate how a POSA would have understood the structure of and the relationship between Bowdish and Evans, right?

A. Yes, that's correct.

Q. This figure starts with h5G1.1 in the upper left-hand corner, right?

A. That's correct.

Q. And that's how you're designating the claimed antibody of the -- of the '149 Patent here,

right?

MS. STERLING: Objection, form.

A. That's consistent with how we're referring to the claimed antibody in the '149 Patent, correct.

Q. Green in this figure means sequences that correspond with the claimed antibody of the '149 Patent, right?

A. That's correct. Maybe I should --

Q. I actually have a different question.

A. Maybe I should clarify that too.

Q. I have a different question, sir.

A. It's also the same antibody as in Bowdish.

Q. Sir, sir, sir, I have a different question. The antibody --

MS. STERLING: Can you let --

MR. DIAMOND: The antibody --

MS. STERLING: -- The witness finish, please.

BY MR. DIAMOND:

Q. The antibody that's in Bowdish is the construct that's on the right-hand side of this figure called Bowdish's 5G1.1-TPO, right?

A. So, again, as we mentioned before, I wouldn't describe this as an antibody, but as a

construct, and the generation of Bowdish's TPO mimetic construct is based on taking antibody --

Q. So, sir --

A. -- that is a (indecipherable) 5G1.1 --

Q. Sir, sir, my question is simply --

A. -- removing the human --

Q. Sir, sir, sir, you're not --

A. -- (inaudible) for CDR3.

Q. Sir, my question is that the construct that's described in Bowdish is the 5G1.1-TPO construct on the right-hand side of this figure, right?

A. So I think that Bowdish refers to both the 5G1.1 version that's on the left portion as well as the 5G1.1-TPO version on the right. So they talk about the version on the left by saying that with a starting antibody scaffold 5G1.1 they remove the CDR3 heavy chain region and replace that with a TPO mimetic. And so both are essentially described within Bowdish.

Q. The construct that's represented by figures 13A and 13B of Bowdish is the figure on the right-hand side of this, right?

A. Yeah, that's right. The figure on the far right, figure 12, is consistent with the sequence

information from 13A and 13B in Bowdish.

Q. The scFv's of Evans were not generated by fragmenting them off a larger antibody, right?

A. The scFv's of the Evans patent are generated by molecular (indecipherable) methods, not by like digestion.

Q. Okay. So the Evans scFv's were not part -- part of a larger Y antibody structure at some point and then removed, right?

A. The scFv protein was something that was generated through methods that are described in Evans, and that sequence relates to CDR regions that are within the 5G1.1 antibody.

Q. But as you said, they weren't broken off from a larger intact antibody, right?

A. Correct. The --

Q. Okay.

A. -- the protein itself was not broken off from a larger protein, but the sequences for the CDR region were taken from --

Q. Well, I'm asking -- that's all I asked you, sir. That's all I asked you, sir.

A. -- the 5G1.1 antibody.

Q. Sir, I'm not asking you -- please, stop answering questions that nobody's asking you.

Okay? Your counsel can ask you questions. You've got to stick with my questions. We have limited time here and this is my one chance to get your testimony. So you've got to stick with my questions.

A. I'm just trying to give a complete answer to your question.

Q. Sir, you're not giving me a complete answer, you're answering a different question. So please listen carefully to my questions. Thank you.

Let's talk about Mueller. Have you got Mueller in front of you, AMG-1008?

A. Yes, I do have AMG-1008.

Q. All right. It's your opinion that Mueller taught methods of developing recombinant antibodies to reduce immune-mediated organ transplant rejection, right?

A. Are you referring to a specific statement within my declaration?

Q. I'm asking you, sir, if it's your opinion that Mueller taught methods of developing recombinant antibodies to reduce immune-mediated organ transplant rejection?

A. I think that's generally consistent with

what I said about Mueller's objective within my declaration, but I prefer to look at it if you're referring to a specific section.

Q. It's really simple. You're sitting here today and I'm asking you what your opinion is.

A. Okay.

Q. Do you understand or do you not understand that Mueller taught methods of developing recombinant antibodies to reduce immune-mediated organ transplant rejection?

A. Great. Let me refer to my declaration to see what I said about Mueller.

Q. Can you not tell me your opinions on Mueller without looking at your declaration?

A. I want to be sure that the answers that I give are completely accurate with respect to the testimony that I've provided through the declaration. Is that a difficulty?

Q. Well, I'm -- I'm trying to understand how this process works here. I mean, you read Mueller in preparing your declaration, right?

A. That's correct. I've read portions of Mueller.

Q. And you reviewed Mueller to prepare for your deposition today, right?

A. That's correct. I've reviewed Mueller as part of the preparation for today's deposition.

Q. So I'm asking you a basic high-level question about Mueller. Okay? You understand that Mueller taught methods of developing recombinant antibodies to reduce immune-mediated organ transplant rejection, right, sir?

(Witness reviewing document.)

Q. Let me ask you this. I mean, if you were providing testimony today before the Board would you agree with that statement?

A. I'm under the impression that today's deposition is equivalent to providing testimony to the Board.

Q. Great. So with the understanding that you're providing testimony before the Board today, can you agree with me that Mueller taught methods of developing recombinant antibodies to reduce immune-mediated organ transplant rejection?

A. Because I know that I'm under oath and interested in being sure that I give completely accurate answers, I would like to review materials before sometimes providing a response and -- so I would do the same -- have the same interest in being accurate no matter what the setting would be.

Q. So you can't answer my question?

MS. STERLING: Objection, misstates the witness's testimony.

A. Yeah. I do believe that I did answer the question.

Q. You haven't answered the question. The question is Mueller -- Mueller taught methods of developing recombinant antibodies to reduce immune-mediated organ transplant rejection, right? Does that sound wrong to you?

A. That is my recollection of Mueller.

Q. All right. And you understand that Mueller provides the sequences of two antibodies that target the P select and protein VCAM as examples, right?

A. Yes, that's my recollection of Mueller.

Q. Okay. Mueller didn't provide the complete sequences for any anti-C5 antibodies, right?

A. No, Mueller did not provide complete sequence to any anti-C5 antibodies, to my recollection.

Q. You review -- you rely on Mueller for the disclosure of sequences relating to hybrid IgG2/G4 constant regions, right?

A. That's correct.

Q. And the sequences -- or let me rephrase that.

Mueller doesn't disclose the sequence of any hybrid IgG2/G4 constant region in the context of an h5G1.1 antibody, right?

A. I don't believe that Mueller gives direct reference to h5G1.1, but the sequence that is disclosed in Mueller is the same sequence as within the h5G1.1 antibody that is known from Sequence ID 2 and Sequence ID 4.

Q. Right. Those are the sequences that you know from -- that are disclosed in the -- in the challenged patents, right?

A. That's correct.

Q. Okay. And with respect to the anti-VCAM antibodies in Mueller that -- that use a hybrid IgG2/G4 isotype, Mueller doesn't expressly identify which sequences correspond to the heavy chain constant region, right?

A. So could you restate your question? Did you say expressly indicates?

Q. Yeah. I said with respect to the anti-VCAM antibodies in Mueller that use a hybrid IgG2/G4 isotype, Mueller doesn't expressly identify which sequences correspond to the heavy chain

constant region of those antibodies, right?

A. Right. They provide sequence that includes the heavy chain constant regions for the G2/G4 construct.

Q. Okay. And in paragraph 121 of your '149 Patent declaration, can you please turn there.

A. I'm sorry. Could you give me the location again.

Q. Paragraph 121 of your '149 Patent declaration.

A. Yes, I'm at paragraph 121.

Q. Great. You state that "Mueller discloses a humanized antibody directed against human C5," right? It's at the top of page 76. You say that "Mueller discloses a humanized antibody directed against human C5," right?

A. Yes, I do see that here.

Q. And you cite -- you have a citation to Mueller here which is column 12 -- or page 12, lines 1 to 2; do you see that?

A. I do see that.

Q. All right. Let's take a look at what Mueller says. You got Mueller in front of you?

A. So AMG-1008 --

Q. 1008, yeah.

A. Yeah.

THE REPORTER: Guys.

Q. And if you could please look at page 12. So do you see lines 1 and 2? Are you up there?

A. Yes, I do.

Q. All right. So Mueller in lines 1 and 2 on page 12 refers to a "Humanized antibody directed against human C5 (h5G1.1 CO12 HuG4 mAb)"; do you see that?

A. I do see that.

Q. Mueller was using that antibody as a control in experiments regarding blocking binding of Ramos to PAEC, right?

A. I see that it was included within this experiment described in figure 11 which relates to inhibition of Ramos binding to PAEC, yes.

Q. And that -- that wasn't a study of C5 binding activity, right?

A. That's correct.

Q. And the antibody that Mueller describes here on page 12 in lines 1 to 2 as being directed against human C5 is a humanized IgG4 isotype antibody, right?

MS. STERLING: Objection, form.

A. So on line No. 2 the antibody that's

referenced with h5G1.1 CO12 HuG4 mAb, my impression of that would be that that was relating to a humanized -- a human G4 construct. There --

Q. And that's what a person --

A. There --

Q. -- of ordinary skill in the art --

A. There are other references on the same page.

Q. Sir, I'm not asking you about that.

A. There's other references on the same page.

Q. Please listen to my question, sir.

A. Okay.

Q. Thank you. Your counsel can ask you questions later. Okay? I'm asking you about page 12, lines 1 and 2. Are you with me?

A. Yes.

Q. Okay. Great. You see an antibody referenced there and it's h5G1.1 CO12 HuG4 mAb, right?

A. I see that referenced at that location.

Q. Great. And Mueller refers to that antibody there, and that's the page and line that we saw you cite on the top of page 76 of your declaration, right?

A. Yeah, it is --

Q. Yes, it is. Okay. Thank you.
A. -- the page that's cited --
Q. All right.
A. -- and elsewhere on the page there's also --
Q. Great. We'll talk --
A. -- a definition of other concepts.
Q. We'll talk -- we can talk about other things. Let's stick with my questions for now. Okay, sir?
Back on Mueller, page 12, lines 1 to 2, okay, Mueller refers to that antibody, h5G1.1 CO12 HuG4 mAb, so Mueller refers to that antibody as a humanized antibody directed against human C5, right?
A. Mueller says in the line that you're referring to "Neither humanized antibody directed against human C5" and then refers to h5G1.1 CO12 HuG4 mAb.
Q. Right. And a person of skill in the art would have understood that that antibody referred to in line 2 on page 12 of Mueller was an IgG4 isotype humanized antibody, right?
A. Did you say line 12? Page 12?
Q. Page 12, line 2, a person of ordinary

skill in the art would understand that the h5G1.1 antibody referred to at that location was an IgG4 isotype antibody, right?

A. I think that that would be an appropriate expectation, yes.

Q. They wouldn't assume that that's an IgG2/G4 antibody, right?

A. No. I think they would probably look to line 30 --

Q. Sir, that's not my question. They wouldn't refer -- they wouldn't -- listen to my question.

A POSA wouldn't have understood that the h5G1.1 HuG4 mAb being referred to on page 12 in line 2 of Mueller is an IgG2/G4 hybrid antibody, right?

A. Yeah. I would think that the reference that you referred to would most likely be interpreted as a G4 humanized antibody.

Q. Thank you.

A. So you --

Q. And let me --

A. I'm sorry.

Q. Sir, sir, my question is finished. I'm asking you another question.

A. But --

Q. Sir, I'm asking you another -- sir, I'm done asking you that question. I have another question for you. Okay? Your counsel can ask you questions later.

My question for you is a person of ordinary skill in the art would understand that this IgG4 isotype antibody referred to in line 2 on page 12 of Mueller is a different antibody from the h5G1.1 G2/G4 antibody that's described elsewhere in Mueller as another isotype control, right?

A. So you did mention that there would be an opportunity as we come to typos to correct them. Is this an appropriate opportunity? This is a typo obviously.

Q. I don't understand what you're saying and --

A. Earlier in the deposition --

Q. I don't -- sir, sir, sir, sir, sir, I'm not asking you a question about typos. I'm asking you what's in your declaration. I'm asking you what's in Mueller. Right now I'm asking you about what's in Mueller. Okay?

A. Uh-huh.

Q. And you understand that this citation in

Mueller on page 12, lines 1 to 2 is talking about an antibody with an IgG4 constant region that's different than an antibody with a G2/G4 constant region, right?

A. Yeah. In giving --

Q. Yes. Okay.

A. -- my answer you had said earlier today when I mentioned that there were some typos --

Q. Sir, sir, sir --

A. -- that I would like to be able to correct, and as we come to them --

Q. If your counsel wants to ask you about typos she can. I'm not asking about typos right now.

A. -- that there would be an opportunity to be able to address them.

Q. Sir, I'm not asking about that. Okay? I'm asking you about what's in your declaration. All right?

That's consistent -- that statement in Mueller on pages -- page 12, lines 1 and 2, that's consistent with the teachings of Thomas that a humanized h5G1.1 antibody with an IgG4 isotype was effective in binding C5 and blocking its activity, right?

A. Could you restate the question?

Q. That teaching in Mueller at the top of page 12, lines 1 to 2 that a humanized antibody -- that -- I'll restate it.

That teaching in Mueller at the top of page 12, lines 1 to 2 that h5G1.1 HuG4 mAb is an antibody directed against human C5 is consistent with the teachings in Thomas that a humanized h5G1.1 antibody with an IgG4 isotype was effective in binding C5 and blocking its activity, right?

A. It's really a comparison that potentially is of apples and oranges. So what's referred to in line 2 of the Mueller reference is designated as h5G1.1 CO12 HuG4 mAb, and my recollection of the Thomas reference is that a different designation is used for that antibody. And so I don't see that it's possible to draw directly a comparison or relationship between results of Thomas with the designation that's here.

Q. So you don't -- let me just make sure I understand what you're saying.

A. Uh-huh.

Q. Are you saying that you don't know if this h5G1.1 IgG4 isotype antibody at the top of page 12 of Mueller is the same h5G1.1 IgG4 isotype antibody

described in Thomas?

A. Yeah. What I'm trying to say is that you've asked me if this teaching on the top of page 12 of Mueller is consistent with what is found within Thomas, and I'm saying it may not be referring to the same construct.

Q. So you think it's possible there may be different humanized IgG4 isotype antibodies that were developed?

A. I do think it's possible that different humanized IgG4 antibodies could be developed, I do.

Q. And based upon the information that's provided in Mueller, you -- you can't tell -- you can't compare these antibodies, right?

MS. STERLING: Objection, form.

A. So I wouldn't want to make the statement that the results that are found in Thomas are consistent with the term that you used to the teaching that's present in Mueller.

Q. Okay. Let's put it this way. Thomas disclosed a humanized 5G1.1 IgG4 isotype antibody, right?

A. That's correct.

Q. And Thomas disclosed that that antibody

was effective in binding C5 and then also worked as potently as the mouse antibody in in vitro assays of complement blocking activity, correct?

A. That's correct. That was shown.

Q. Okay. And the antibody that's referred to on page 12 of Mueller in line 2 is -- is a -- or in lines 1 and 2 is a h5G1.1 antibody with an IgG4 isotype, right?

A. That would be my understanding of the reference at the top of page 12 of Mueller.

Q. Right. And Mueller says that that IgG4 isotype h5G1.1 antibody was a humanized antibody directed against human C5, right?

A. Yes, it does say that.

Q. Okay.

Mueller doesn't contain any citations to Evans, right?

A. So Mueller was published in 1997 and it predates Evans. So I'd be confident in saying there would be no citations to Evans.

Q. And in -- in your declarations in this case sitting here today you're not aware of anything where you point to any statements in Mueller that characterize an h5G1.1 antibody with a hybrid IgG2/G4 isotype as having C5 binding or C5

cleavage blocking activity, right?

A. So I -- I think on page 12 the reference to h5G1.1 CO12 HuG2/G4 mAb is reference to an antibody that has a humanized G2/G4 that is derived from the anti-C5 antibody known as 5G1.1.

Q. Sir, you've got to listen to my question. Is there anything in Mueller that states that the h5G1.1 HuG2/G4 mAb used as an isotype control has C5 binding activity? And if there is, please point me to where that is in Mueller.

(Witness reviewing document.)

Q. And Mueller is a long document. So, again, given the limited time that we have today to talk about all the patents and all the issues, I don't want to burden you with looking through all of Mueller all over again.

Let me ask you first, have you ever read through Mueller from cover to cover?

A. No. The reason why I'm looking is that the antibody designation of 5G1.1 is known as an anti-C5 antibody, and it's likely that within Mueller there would be a reference to humanized 5G1 antibodies that are anti-C5 antibodies being represented --

Q. Well, that's just speculation. You're

just guessing that it's in Mueller, right? You don't know?

A. So I would like to look through it carefully to be able to give a good answer to your question, and so that's why I'm looking --

Q. Did you look through it carefully to prepare your declarations?

A. I looked through for the portions that were referred to and it -- you're asking me about a specific reference to a specific phrase, and I don't know the --

Q. I'm asking about your testimony in this case, sir. Let's go back to your declaration.

A. -- document --

Q. Look at your declaration.

A. -- line by line to be able to answer.

Q. Sir, go to your declaration, paragraph 121.

A. Okay.

Q. Okay. Let's go back to that. You represented in sworn testimony on page 76 that Mueller discloses a humanized antibody directed against human C5, right?

A. That's correct.

Q. Then you cited to an IgG4 isotype antibody

there, right?

A. Yeah. As we mentioned before, there were some typos that I asked to correct at the beginning of the dep- --

Q. So you think that's a typo?

A. Yes.

Q. Okay.

A. Because on page 12 there is also a reference to a construct that is a G2/G4.

Q. But on page 12 that reference that you're referring to, lines 35 to 37 on page 12?

A. Uh-huh.

Q. It doesn't mention C5 binding there, does it?

A. No. It mentions, though, that it's through the --

Q. Sir, no, no. My question is it doesn't mention C5 binding there on page 12 at lines 35 to 37 for the human G2/G4 construct, right?

A. So humanized G5 --

Q. Sir, sir, does it say -- does it say -- look at page 12, lines 35 to 37. Does it mention C5 binding there for that G2/G4 construct, yes or no?

A. Humanized --

Q. Sir.
A. -- 5G1.1 antibodies --
Q. Sir, that's not a --
A. -- have that activity.
Q. -- that's not -- that's not a yes-or-no answer. The question is --
MS. STERLING: I'm sorry. Can you let him get the answer out and then determine --
MR. DIAMOND: No, because he's not answering my question.
MS. STERLING: -- an answer.
BY MR. DIAMOND:
Q. You're not answering my question. My question is you do not see any mention on page 12, lines 35 to 37 that the h5G1.1 G2/G4 construct used as an isotype control here has C5 binding activity? That's not mentioned here, right?
A. That's correct.
Q. Okay. Thank you.
Let's look at -- there's no clinical data reported in Mueller that you know of, right? Sitting here today without reading through all of Mueller and all of your declarations, you're not aware of any clinical data in Mueller, right?
A. No, I don't recall any data that relates

to activity in patients.

Q. And you're not aware of any data in Mueller regarding safety or immunogenicity of the IgG2/IgG4 constructs, right?

MS. STERLING: Objection, form.

A. No specific recollection of that.

Q. Okay. You don't recall including that in your declarations, right?

A. That's correct.

Q. Okay. In paragraph 54 of your declaration --

A. The '149 declaration?

Q. Your '149 declaration, yeah. Actually, no. Let's -- let's move on from that. I want to get these two, 1029 and 1030.

MR. DIAMOND: These are for the witness. Thank you.

THE WITNESS: Thank you.

BY MR. DIAMOND:

Q. So do you have AMG-1029 and AMG-1030 in front of you?

A. Yes, I do.

Q. All right. AMG-1029 is the Wang 2006 -- or I guess Wang 2007 reference that you rely on in this case, right?

A. That's correct.

Q. And AMG-1030 is the Daugherty, et al., reference that you rely on in this case?

A. That's correct.

Q. You mention in your declarations that Wang, 1029, and Daugherty, 1030, include tables regarding the various commercialized monoclonal antibody products that existed as of March 15th, 2007 and their pharmaceutical compositions, right?

A. I think that's correct. Would you direct me to the portion of the declaration that you're referring to?

Q. Sure. So, for example, if we go to your '880 declaration, you can look at paragraph 106 of that.

A. Yes, I'm at that paragraph.

Q. And specifically I'm directing you to the bottom of page 72 bridging onto page 73 of your '880 declaration where you say "My review of approved therapeutic antibodies approved by the FDA before March 15th, 2007" and it -- and it goes on and you're citing AMG-1029 table 1 and AMG-1030 table 1; do you see that?

A. Yes, I do.

Q. So the two documents and the tables that

you reference here for your review of therapeutic antibodies approved by the FDA before March 15th, 2007 are these two references, the Wang 1029 reference and the Daugherty 1030 reference, and their table 1's, right?

A. Those are some of the references that I reviewed that fit that description that you mention, but there's many others as well that are listed within the declarations. If you look at the publication list for the references that go from -- so AMG-1056 through AMG-1076 inclusive would also relate to that same topic area, information that relates to approved antibodies.

Q. And generally you understand there would be overlap between that collection of references from 1056 onward and the tables that -- that collect the products in these two documents, 1029 and 1030?

A. I agree there's overlap between the two.

Q. Okay. Well, let's -- let's look at 1029 and go to table 1 of that document. You'd agree that none of the -- none of the antibodies or -- or compositions described in table 1 of AMG-1029 features an antibody with a hybrid IgG2/IgG4 constant region, right?

A. You know, I am not a hundred percent certain of that, but that's my impression.

Q. Sitting here right now, you're not aware of any products approved prior to March 15th, 2007 that used a hybrid IgG2/G4 constant region, right?

A. Yes, that's correct.

Q. All right.

If you go to the first page of AMG-1029, you see three sentences -- have you got the abstract there? Do you see that?

A. Sorry. AMG-1029 --

Q. 1029 -- 1029 is the abstract

THE REPORTER: Guys.

A. I do see the abstract.

Q. There's an abstract and three sentences down you see it begins "Although"?

A. Yes, I do see that sentence.

Q. So Wang states here that "Although antibodies share certain structural similarities, development of commercially viable antibody pharmaceuticals has not been straightforward because of their unique and somewhat unpredictable solution behavior"; do you see that?

A. I do see that.

Q. All right. And if you go to page 5 of

Wang, AMG-1029, looking at the upper left-hand corner you see it says "The development of commercially viable antibody pharmaceuticals has, however, not been straightforward. This is because the behavior of antibody seems to vary even though they have similar structures," right?

A. I do see that sentence within the reference.

Q. Okay. And if you go to page 8 of AMG-1029 in the lower right-hand corner, you see it states -- there's a section on aggregation; do you see that?

A. I do see that.

Q. And it says "Antibody aggregation is a more common manifestation of physical instability," right?

THE REPORTER: Please slow down. I cannot understand you.

Q. It says "Antibody aggregation is a more common manifestation of physical instability"; do you see that?

A. I do see that sentence.

Q. It says "The concentration-dependent antibody aggregation was considered the greatest challenge in developing protein formulations at

higher concentrations"; do you see that?

A. I do see the reference to higher concentrations.

Q. And just going back for a second to -- do you have Kaplan in front of you, AMG-1021?

A. Yes, I do see that.

Q. I've got it here. I got it.

We were looking earlier at page 2, the section on the right-hand column -- well, sorry. It's page 1028, "Synthesis and SAR"; do you see that?

A. Is it 1018?

Q. 1018.

A. Yeah, I do see that.

Q. "Synthesis and SAR" on the right-hand side; do you see that?

A. Yes, I do.

Q. And you see a few lines up from the bottom of that paragraph the sentence that begins "5G1.1 is supplied"; do you see that?

A. Yes, I do.

Q. And it says "5G1.1 is supplied as a sterile, nonpyrogenic solution (2 milligrams per milliliter) for IV injection," you see that, right?

A. I do see that.

Q. And sitting here right now, you don't recall discussing that in your declarations, right?

A. Discussing this reference to Kaplan or the antibodies within the range let's say of 1 to 30 mgs per ml would be appropriate range in (indecipherable).

Q. This reference to Kaplan that we just read, you don't reference that in your declarations, right?

A. So I think Kaplan is referred to in the declaration. Do you mean this specific portion of Kaplan?

Q. This specific portion of Kaplan that refers to the statement "5G1.1 is supplied as a sterile, nonpyrogenic solution (2 milligrams per ml) for IV injection," you don't refer to that in your declarations?

A. That's correct.

MR. DIAMOND: Okay. I think we should take a break and figure out the rest of the day.

THE VIDEOGRAPHER: We're going off the record. The time is 17:02.

(A short break was had.)

THE VIDEOGRAPHER: Going back on the record. The time is 17:26.

BY MR. DIAMOND:

Q. Dr. -- Dr. Balthasar, how many antibody formulations have you developed?

A. So we've developed in my lab and used probably hundreds of antibodies and have formulated them in many cases for administration to subjects that we were studying. So in that context I have been involved with and my research group has been involved with formulating many antibodies.

Q. And how many of those were commercialized?

A. So none of the antibodies that we've developed from our work have led to commercialized products, but we've worked with many commercial antibodies and some of the work that we've done has led to patents for antibodies directed against particular targets that have progressed to late-phase clinical studies now.

Q. So you haven't developed any formulations of antibodies that have been commercialized, right? I'm talking about -- let me rephrase that. You haven't developed with any pharmaceutical compositions of antibodies for human use that have been commercialized, right?

MS. STERLING: Objection, form.

A. Yeah. So we have not developed a unique

composition that has been used -- you know, as developed in our lab that has been used in humans.

Q. Have you ever developed an antibody composition for nebulization?

A. So I have not done any work with nebulization but have worked with antibodies in solution that would be of similar nature to that use in nebulization.

Q. You haven't developed any antibodies in nebulization?

A. That's correct.

MR. DIAMOND: This is the next one, 1028.

BY MR. DIAMOND:

Q. So this is AMG-1028, the Wang patent application that you rely on in this case, right?

A. That's correct, yep.

Q. Wang doesn't disclose the sequence of eculizumab, right?

A. I don't believe there's any information on the sequence of eculizumab within this patent application.

Q. Wang doesn't include any disclosure that eculizumab -- or I guess Wang doesn't have any disclosure as to what the heavy chain constant

region of eculizumab is, right?

A. So eculizumab is a name associated with a sequence --

Q. I'm not asking you, sir, I'm not asking about what eculizumab as a name and where it goes. I'm asking you about looking at Wang, the document, do you have that in front of you?

A. I do.

Q. There's nothing in Wang that states what the identity of the heavy-chain constant region of eculizumab is, right?

A. Not beyond the name eculizumab.

Q. Wang -- nothing in Wang says what the structure of the heavy-chain constant region is for the antibody with the name eculizumab, right?

A. Yeah. There's nothing, to my recollection, that gives any information about the structure of eculizumab --

Q. Okay.

A. -- beyond the name eculizumab.

Q. Okay. Show me where the name eculizumab appears in Wang. Actually, never mind. Never mind.

Let me ask you this. The title of Wang is "Nebulization of Monoclonal Antibodies for Treating

Pulmonary Diseases," right?

A. That's the title, yes.

Q. PNH isn't a pulmonary disease, right?

A. That's correct.

Q. Nebulized formulations are for oral inhalation, right?

A. They're for inhalation.

Q. Or it could be nasal inhalation, right?

A. Yes.

Q. You're not aware of any teaching in the prior art that PNH could be treated by administering a drug by inhalation, right?

A. I'm not aware of any information in the prior art that refers to treatment of PNH by nebulization, but this reference is really (inaudible) --

Q. Sir, sir, sir, that was my question. That was my question. Okay?

And you rely in part on a figure 10 in Wang, right?

A. That's correct.

Q. And if we turn to -- it's the page with page No. 20 at the bottom of Wang, 1028. The page number at the top is 3. Let me know when you're there. You have to go in past all the figures and

stuff.

A. Yeah.

Q. It's the page with paragraph No. 25 in the lower left-hand corner. Are you there?

A. Yes, I see that.

Q. And it states here in Wang that figure 10 shows exemplary formulations suitable for nebulization of an antibody, right?

A. That's what it says, yes, that's correct.

Q. Wang doesn't expressly disclose any 300-milligram single-use dosage forms, right?

A. Yeah. I'm not aware of any discussion of a 300-milligram single-use dosage form within Wang.

Q. And there's no specific discussion in Wang of a 10-milligram-per-milliliter formulation for intravenous infusion, right?

A. Yeah. I just see, for example, in figure 10 under formulation 1 antibody concentrations ranging from 1 to 30 mgs per ml in solution compositions that are consistent with what one would usually use for -- or with intravenous.

Q. Sir, that's the -- that's the figure that we saw was identified as exemplary nebulization compositions, right?

A. Yes.

Q. Okay.

A. It's showing compositions that are appropriate for nebulization, which is in aqueous solution.

Q. Sir, there's no example or specific -- there's no example in Wang of a 10-milligram-per-milliliter composition for intravenous infusion, right?

A. No, there's no example in Wang that shows a 10-milligram-per-ml solution for intravenous administration, just appropriate compositions with consideration I think of things like solubility and stability.

Q. In your view prior to March 15th, 2007 was there a need in the medical field for -- for an approved treatment of PNH?

MS. STERLING: Objection, form.

A. So maybe could you restate that? So was there a need for an approved treatment of PNH at the time of 2007; is that the question?

Q. At the time of March 15th, 2007 was there a need for an approved treatment of PNH?

MS. STERLING: Objection, form.

A. I think that PNH was a condition that would benefit from development of new treatment.

So in that context there, you know, could be a need for new treatment for that disease.

Q. And at that time there was a need for an approved treatment of that disease, right?

MS. STERLING: Objection, form.

A. Yeah. I think that that is a statement that is appropriate.

Q. Okay. Have you considered the sales of Soliris?

MS. STERLING: Objection, scope.

A. So considered in what way?

Q. In the context of forming your opinions in this case, have you considered the sales of Soliris?

MS. STERLING: Same objection.

A. Not maybe directly with respect to the opinions that are made within the case.

Q. Okay. And you don't discuss the sales of Soliris in your opinions in this case, right?

A. Let me refer back. I don't recall making a direct statement about sales of Soliris, but I just want to be positive that there's no such reference within -- I don't make any statement with respect to sales, to my recollection, throughout the -- no, there's nothing in the declarations that

I recall.

Q. You understand that the antibody that's recited in the -- the various challenged claims that we looked at, the antibody with a heavy chain of Sequence ID No. 2 and a light chain of Sequence ID No. 4, that is the antibody that's the active ingredient in the Soliris product, right?

MS. STERLING: Objection to the extent it calls for a legal conclusion.

MR. DIAMOND: Counsel, that's coaching and doesn't even make any sense.

You can answer my question

A. So you're asking me if the product marketed as Soliris in 2007 is the same product as associated with Sequence ID 2 and 4, and the answer is yes.

Q. Okay. And the same -- it's still the same product, to your knowledge, today, right?

A. Yeah. And I think that's completely consistent with things in the declaration, for example, the Boone declaration.

Q. Thank you. Thank you. Thank you.

In your declarations you state that researchers were -- were actively developing therapeutics that targeted the complement system as

treatment for PNH long before March 15th, 2007, right?

A. That's correct.

Q. But as far as you're aware, prior to the filing of the challenged patents at issue in this case, no one other than Alexion had developed such a product successfully, right?

MS. STERLING: Objection, form.

A. So I'm trying to understand your question. So you're saying --

Q. I'll rephrase it. I'll rephrase it.

You're not aware of anyone other than Alexion prior to March 15th, 2007 having shown the safety and efficacy of an anti-C5 antibody for the treatment of PNH, right?

MS. STERLING: Objection, form.

A. So I feel that -- I don't have a clean answer to that question because many of the individuals, to my knowledge, who are associated with the clinical studies that showed -- demonstrated safety and efficacy, to my knowledge, were not Alexion employees.

Q. Okay. Let's -- for purposes of this question when I say Alexion, let's understand I'm referring to Alexion and any, you know, researchers

that Alexion worked with in the context of supporting its clinical research. Okay?

A. Okay.

Q. Aside from that -- that group of people, Alexion and the researchers that it worked with, you're not aware of any other research group prior to March 15th, 2007 that demonstrated the safety and efficacy of an anti-C5 antibody for the treatment of PNH, right?

A. Yeah. Off the top of my head I can't recall seeing any other demonstration of clinical safety and efficacy of an anti-C5 antibody for treatment of PNH.

Q. Okay.

Who have you had -- again, I'm not asking for the contents of any specific communications, right, but who have you had contact with at Sterne Kessler?

A. The names of all the individuals?

Q. Yes, please.

A. To David Holman, Deborah Sterling, David Roadcap are prime people who I've had contact with.

Q. Who first contacted you about this case?

A. Honestly, I don't recall.

Q. Do you think it was one of the individuals

in this room?

A. It could have been David Roadcap. I honestly don't remember. He's not here in the room today.

Q. And when was that?

A. So my recollection would be that that would be sometime in 2018.

Q. Uh-huh. And was your -- was your first meeting with Sterne Kessler a phone call?

A. Yeah. The first meeting that I had I guess if -- so we had e-mail exchanges, then we had telephone calls.

Q. And aside from the individuals that you've mentioned so far at Sterne Kessler in between that initial call and through to today, were there any other individuals, attorneys at Sterne Kessler who you interacted with?

A. You know, honestly, I'm not very good at recalling names. I don't know. The individuals I mentioned I have strong recollection of contact with.

Q. Did you -- have you talked with Eldora Ellison in the context of this case?

A. It's possible.

Q. Okay. Is it possible you talked with Eric

Steffe?

A. Possible.

Q. Okay. What about Cynthia DeRenzo?

A. It's possible. You know, the names don't ring a bell, but it's possible.

Q. You don't know. Is it possible you talked with Marsha Gillentine?

A. It's possible.

Q. What about Trey Powers?

A. I don't recall the name, you know.

Q. If any of them particularly ring a bell, please let me know.

Did you talk with Rebecca Stevens in this case?

A. Not that I have specific recollection.

Q. Okay. What about Jason Eisenberg?

A. Not that I have specific recollection.

Q. Pauline Pelletier, did you talk with her in this case?

A. Not that I have specific recollection.

Q. What about Christopher Gallo?

A. Not that I have specific recollection.

Q. How about Lori Brandes?

A. Not that I have specific recollection.

Q. What about Prati Khanduri?

A. Not that I have specific recollection.
Q. Did you talk with Sean Lou?
A. Not that I have specific recollection.
Q. Or Robert Millonig?
A. Not that I have specific recollection.
Q. Just to -- going back over the names that we just listed, I said talked, did you talk with them, but do you recall having any e-mail communications with those individuals?
A. Yeah, I mean, it's possible. I have to say that, you know, we started talking about this 2018, we filed the declarations in February of 2019, and that's a long time ago for me. So I don't have any specific recollection aside from, you know, the individuals who I mentioned, David Holman, Deborah Sterling, David Roadcap.
Q. No. We talked about some of the references that you've looked at and where you got them. Did you get Bowdish from counsel?
A. Bowdish, yes, did come from counsel.
Q. Okay. And Wang, the 1028 Wang that we were just looking at about compositions, did you get that from counsel?
A. Yes, that did come from counsel.
Q. And both of the Mueller documents that

we've looked at, did those come from counsel?

A. To the best of my recollection.

Q. Okay. Are there any other documents in terms of, you know, prior art that you cited in this case that you can recall that you got from counsel?

A. So I would say that the documents cited came from counsel and documents that I knew of in the past have asked counsel to get. The majority that are listed were documents that were identified from counsel.

Q. And just going back to what we were just talking about earlier, aside from Mr. Holman and Ms. Sterling and Mr. Roadcap, have you had any communication with anyone else at Sterne Kessler that you can recall?

A. Attorneys at Sterne Kessler?

Q. Yeah.

A. Yeah. I don't have specific recollection of other individuals. It's possible, but I just don't recall names and don't have any specific recollection.

MS. STERLING: I'll just be clear, it's Dr. Roadcap, Dr. Holman, and Dr. Sterling. Thank you.

MR. DIAMOND: Sorry to deprive you all of your titles.

BY MR. DIAMOND:

Q. Have you reviewed -- did you review any of Amgen's European submissions prior to preparing your declarations in this case?

A. There was some information that related to processing in Europe that was considered within the declaration.

Q. Do you recall -- do you recall which -- which of Amgen's European submissions you considered in the context of forming your opinions?

A. So information that related to formulation from Dr. Treuheit and Dr. Miano was through contacts of I think the European processing. I think that's the main content that relates to European processing that -- that was considered for the declaration.

Q. Okay. Do you do any other work for Amgen aside from your involvement in this case?

A. I have in the past consulted for Amgen. Amgen is a sponsor for a center that I direct at University at Buffalo. So we have a consortium that's called the Center for Protein Therapeutics. Most of the major pharmaceutical companies involved

with antibody development sponsor the center, including Amgen.

Q. And aside from the work that is -- involves the Center for Protein Therapeutics, have you consulted with Amgen in other capacities?

A. I have. Not recently, but I have in the past.

Q. And if you're able to share, what was the general subject matter of those consultancies?

A. So relating to my expertise in antibody pharmacokinetics and pharmacodynamics, so we did have one collaborative publication with investigators at Amgen.

Q. And do you recall how much you received in compensation for that -- those consultations?

A. It was, I mean, over probably the last 15 or so years. I don't recall. I have no --

Q. Do you know anyone who takes Soliris?

A. No. I, you know, don't have any direct knowledge. There could be people who take Soliris who I know, but I don't have any direct knowledge of anyone who takes Soliris.

Q. Do you know anyone who has PNH?

A. It's possible I know somebody who has PNH, but I don't have any direct knowledge of anyone

having PNH.

Q. Do you know anyone who has atypical hemolytic uremic syndrome?

A. You know, not to my knowledge. It's possible.

Q. Do you know anyone that has myasthenia gravis?

MS. STERLING: Objection, relevance.

A. It's possible. I think I have met somebody who has myasthenia gravis, but I can't -- don't have a specific recollection.

Q. Sure. Do you know if they were taking Soliris?

A. I don't. I don't know.

MR. DIAMOND: I'm going to take a break just to see if there's any remaining questions.

MS. STERLING: Okay.

MR. DIAMOND: Hopefully we can wrap up pretty soon.

MS. STERLING: Okay.

THE VIDEOGRAPHER: We're going off the record. The time is 17:47.

(A short break was had.)

THE VIDEOGRAPHER: We're going back on the record. The time is 18:00 hours.

MR. DIAMOND: Two documents for the witness. Thank you.

BY MR. DIAMOND:

Q. So, Dr. Balthasar, you have in front of you AMG-1019 and AMG-1020, which are two of the documents you rely on in these proceedings, right?

A. That's correct.

Q. And these are -- these are two documents that you rely on for the proposition that there was literature in the art prior to March 15, 2007 that used the term 5g1.1 to refer to eculizumab, right?

A. I don't know. Could you refresh my memory with respect to specific areas of the declaration that make that connection?

Q. Sure. So we could look at your '880 declaration, for example --

A. Okay.

Q. -- at paragraph 46, and you state here in paragraph 46 that "In some publications before March 15th, 2017 the term 5G1.1 was used to refer to the parental murine 5G1.1 antibody," and then you say "but 5G1.1 was also regularly used to refer to eculizumab." You see that, right?

A. Yes, I do.

Q. And you go on to -- on page 33, the next

page, do you see in the last few lines of this paragraph you cite three documents, AMG-1019, AMG-1020 and AMG-1021, right?

A. I do see that. Thank you.

Q. And so you understand that you're citing AMG-1019 and AMG-1020 in this case as documents that identify eculizumab with the term 5G1.1, right?

A. Yes, that's correct.

Q. And you have quotations from -- from each of these documents in your paragraph 46 of your '880 declaration?

A. Yes, that's correct.

Q. So let's look at 1019 first and turn to page 56 that you cite, and we see -- if you look on the left-hand column towards the bottom, you cite -- or the statement that you quote from begins with "Eculizumab (5G1.1"); do you see that?

A. Yes.

Q. And the full statement reads "Eculizumab (5G1.1, Alexion Pharmaceuticals) is a humanized monoclonal antibody that prevents the cleavage of human complement component C5 into its pro-inflammatory components" citing reference 31; do you see that?

A. Yes, I do.

Q. And if you go into the references of this document, which are on page 61 of AMG-1019, you see that reference 31 is the Kaplan document that we looked at earlier as AMG-1021, right?

A. Yes, I see that.

Q. If you look at AMG-1020, you see -- well, let's turn to page 17 of AMG-1020 that you cite.

A. I'm sorry. You said page 17? Do you mean 2123?

Q. I might be in the wrong number here. It's page -- I'm looking here. It's page 2123.

A. Yes.

Q. Yeah. Thanks.

So you're in AMG-1020 at page 2123, right?

A. Yes, that's right.

Q. And you see in the middle paragraph on the right-hand side several sentences down the statement "Eculizumab (5G1.1)" and it goes on?

A. Yes.

Q. And you see that at the end of this paragraph -- well, let me -- let me -- let me -- let me state it this way. So the full sentence is "Eculizumab (5G1.1), the humanized anti-C5 mAb, is considered a potential treatment for several

chronic inflammatory diseases, including rheumatoid arthritis, nephritis, and phase 2 trials have been initiated for these indications," right?

A. I see that, yes.

Q. And there's another sentence that says "Furthermore, eculizumab has been shown to prevent C5 activation in humans and to have beneficial effects in patients with paroxsymal nocturnal hemoglobinuria. Specifically, it reduces intravascular hemolysis hemoglobinuria and the need for transfusion in these patients providing a proof of concept that blockade of complement activation is feasible and tolerable in patients with chronic disease"; do you see that?

A. Yes.

Q. And for that it's citing reference 18, right?

A. Yes, that's correct.

Q. And in reference 18 -- if you go to page 2124 of AMG-1020, reference 18 is the Hillmen 2004 article that we've looked at today as AMG-1004, right?

A. That's correct.

Q. I'm going to show you a picture of the -- of the Web bio of Eldora Ellison from Sterne

Kessler. Do you recognize her?

A. No, I do not.

Q. And I'm going to show you pictures of Eric Steffe of Sterne Kessler from his Web bio. Do you recognize him?

A. No, I do not.

Q. How did you prepare for your deposition today?

A. So I had a couple teleconferences with counsel leading up to today, and then yesterday and the day before I reviewed the declarations with counsel to prepare.

Q. And aside from reviewing the declarations, did you review any other documents?

A. No. Declarations and the references contained within the declarations was the focus of the preparation.

Q. Okay. And which counsel did you have teleconferences with to prepare for your declaration -- your deposition today?

A. Yeah. So the main person that I contacted to prepare for the deposition was David Holman. So he arranged to be able to find convenient times and was the main person I talked to with both of those TC's.

Q. And was there anyone else on the phone during your deposition preparation teleconferences?

A. I think that there were additional people on the phone. I think Angie was on the phone for one of the times. I don't know of everyone who was there, though.

Q. Is there anyone else you can recall who was on those calls for your deposition preparation?

A. Not a hundred percent. Deb, were you there? I don't remember. So I don't recall exactly.

Q. Did you say you had an in-person meeting to prepare for your deposition?

A. So yeah. Yesterday and the day before I did meet with counsel to prepare.

Q. And who were the counsel that were at your in-person deposition preparation meetings yesterday and the day before?

A. So everyone here in the room and at lunchtime David Roadcap stopped in to say hi.

Q. So all the attorneys in this room and David Roadcap, right?

A. Yeah. The attorneys that are on, you know, the Amgen side.

Q. On that side of the room.

And then aside from those attorneys, was there anyone else who was at your deposition preparation?

A. Yeah. No.

Q. How long did you spend each day preparing?

A. So I think, you know, in total it was probably in the range of 20 hours.

Q. Okay. Does that include your teleconferences and the in-person meetings?

A. No. So the teleconferences were an additional two hours, and I did some document review additionally as well.

Q. Okay.

A. I think the total time for deposition preparation was approximately 30 hours.

Q. Okay.

MR. DIAMOND: So we have -- we're ending our questions for the moment, but we consider the deposition open because we reserve the right to recall the witness in view of his conduct today.

MS. STERLING: I'm sorry. Can you repeat that. I didn't follow.

MR. DIAMOND: Sure. We are not asking any further questions right now, but we reserve the right to recall the witness in view of his conduct

today. If you have any questions, go ahead.

MS. STERLING: Okay. Thanks. Let's take a few minutes off the record. Thank you.

THE VIDEOGRAPHER: We're going off the record. The time is 18:11.

(A short break was had.)

THE VIDEOGRAPHER: We are going back on the record. The time is 18:16.

EXAMINATION

BY MS. STERLING:

Q. Dr. Balthasar, I have a few questions for you.

Earlier today near the start of your deposition you mentioned that there were a few typos that you had identified I believe you said yesterday in your declaration. Do you recall that testimony?

MR. DIAMOND: Objection, leading.

A. I did.

Q. I'm sorry. Do you recall that testimony?

A. I did.

MR. DIAMOND: Objection, leading.

BY MS. STERLING:

Q. Did you get an opportunity to fix those typos as the conversation went on with Mr. Diamond?

MR. DIAMOND: Objection, leading.

A. No, I didn't have that opportunity.

Q. Would you like to take the opportunity to correct those typos?

A. I would.

Q. Please do.

A. Yeah. So the --

MR. DIAMOND: Objection, outside the scope of his -- of his declarations in this case and outside the scope of the direct exam.

MS. STERLING: I believe the discussion of typos were a part of the direct. Please go ahead and answer.

A. So the typos that I wanted to correct appear in a couple places within the declarations that I prepared. The testimony is similar across the declarations. So I'd like to refer to the declaration for the '504 Patent and to paragraph 213 and 214 for that declaration. So these are, as we mentioned earlier today, just minor typographical-type errors that I wanted to correct.

So in paragraph 213 I indicated that "I also note" -- in the first sentence "I also note that Alexion has argued before the European Patent Office that the 10-milligram-per-kilogram

concentration offers unexpected stability." So 10-milligrams-per-kilogram is not a concentration. This was a typo and what was intended here is 10-milligrams-per-ml. So just a clerical error.

Then also in paragraph 214 in referring to Dr. Treuheit's testing I had made another typo in this section of the declaration where it refers to "Maintaining over 98 percent monomer percentage," and this is six lines down within paragraph 214. So this was a statement I was making in reference to the World Health Organization or WHO standard of 5 percent or less aggregation. I meant to indicate 95 percent monomer percentage but failed to notice that the declaration said 98 percent.

MS. STERLING: Okay. We have no further questions. Thank you.

FURTHER EXAMINATION

BY MR. DIAMOND:

Q. Just a few questions.

Dr. Balthasar, prior to being asked those questions from your counsel just now did you hear the questions that you were going to be asked?

MS. STERLING: Objection to the extent it gets to privilege. I'm going to instruct you to not answer about the substance of any of our

conversation during that break.
BY MR. DIAMOND:
Q. Actually, did you talk about the substance of the deposition during your last break?
MS. STERLING: You may answer yes or no, but not discuss any substance.
A. We did discuss the interest that I had to correct these typos in paragraph 213 and 214.
Q. Well, I think, Counsel, he's opened the door to talk about what he talked about during the break?
MS. STERLING: No, he hasn't. Those are privileged conversations.
MR. DIAMOND: Those are not privileged conversations during the deposition.
MS. STERLING: They are. You closed the record, you were finished with your cross-examination.
MR. DIAMOND: No, no, no, the deposition was still pending. You can't discuss your redirect with him.
MS. STERLING: You told him you were done questioning him.
BY MR. DIAMOND:
Q. Yes or no, did you discuss your redirect

questions with your counsel prior to returning from the last break?

MS. STERLING: I'm going to instruct the witness to not answer that question.

MR. DIAMOND: You're going to instruct the witness not to answer yes or no?

MS. STERLING: I'm going to instruct -- yes, because you're asking about specific questions. I'm going to instruct the witness to not answer. You can ask more general questions, but please don't get to the substance.

BY MR. DIAMOND:

Q. Did you discuss redirect examination during your last break?

MS. STERLING: You may answer yes or no.

A. Yes.

MR. DIAMOND: Counsel, we're entitled to talk to the witness about -- about the substance of your communications with him during -- during -- during the break.

MS. STERLING: You're not, not during this break. Any other break you're entitled to and you asked that. During this break, no. The Board has been clear that between the close of cross-examination and redirect those conversations are

privileged.

MR. DIAMOND: We disagree, but if you're going to --

BY MR. DIAMOND:

Q. Are you going to follow your counsel's instruction?

A. Yes.

MR. DIAMOND: All right, then, we reserve the right to raise that with the Board.

MS. STERLING: You can go ahead. The Board will point you to their decisions on that point.

MR. DIAMOND: I disagree.

No further questions.

MS. STERLING: Okay.

THE VIDEOGRAPHER: This concludes the deposition of Dr. Joseph Balthasar. We're going off the record at 18:21.

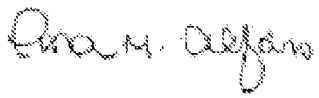
(Whereupon, at 6:21 p.m. the taking of the instant deposition ceased.)

CERTIFICATE OF SHORTHAND REPORTER-NOTARY PUBLIC

I, TINA M. ALFARO, Registered Professional Reporter, Certified Realtime Reporter, and Notary Public, the officer before whom the foregoing deposition was taken, do hereby certify that the foregoing transcript is a true and correct record of the testimony given; that said testimony was taken by me stenographically and thereafter reduced to typewriting under my direction; that reading and signing was requested; and that I am neither counsel for, related to, nor employed by any of the parties to this case and have no interest, financial or otherwise, in its outcome.

IN WITNESS WHEREOF, I have hereunto set my hand this 25th day of October, 2019.

My Commission Expires: October 31, 2020



NOTARY PUBLIC IN AND FOR THE
DISTRICT OF COLUMBIA

Deborah Sterling, Ph.D., Esquire

October 25, 2019

RE: Amgen Inc. v. Alexion Pharmaceuticals, Inc.
10/23/2019, Joseph P. Balthasar , Ph.D. (#3564685)

The above-referenced transcript is available for review.

Within the applicable timeframe, the witness should read the testimony to verify its accuracy. If there are any changes, the witness should note those with the reason, on the attached Errata Sheet.

The witness should sign the Acknowledgment of Deponent and Errata and return to the deposing attorney. Copies should be sent to all counsel, and to Veritext at cs-midatlantic@veritext.com

Return completed errata within 30 days from receipt of testimony.

If the witness fails to do so within the time allotted, the transcript may be used as if signed.

Yours,

Veritext Legal Solutions

Amgen Inc. v. Alexion Pharmaceuticals, Inc.

Joseph P. Balthasar, Ph.D. (#3564685)

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Joseph P. Balthasar, Ph.D. Date

Amgen Inc. v. Alexion Pharmaceuticals, Inc.

Joseph P. Balthasar , Ph.D. (#3564685)

ACKNOWLEDGEMENT OF DEPONENT

I, Joseph P. Balthasar , Ph.D., do hereby declare that I have read the foregoing transcript, I have made any corrections, additions, or changes I deemed necessary as noted above to be appended hereto, and that the same is a true, correct and complete transcript of the testimony given by me.

_____ _____

Joseph P. Balthasar, Ph.D. Date

*If notary is required

SUBSCRIBED AND SWORN TO BEFORE ME THIS

_____ DAY OF _____, 20___.

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The Oncologist®

Regulatory Issues: FDA

FDA Report: Eculizumab (Soliris®) for the Treatment of Patients with Paroxysmal Nocturnal Hemoglobinuria

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Key Words. Eculizumab • Paroxysmal nocturnal hemoglobinuria • PNH • Drug approval

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LEARNING OBJECTIVES

After completing this course, the reader will be able to:

1. Describe the mechanism of action of eculizumab in PNH.
2. Discuss the efficacy findings upon which the approval of eculizumab was based.
3. Manage the safety concerns surrounding the use of eculizumab for the treatment of patients with PNH.

CME This article is available for continuing medical education credit at CME.TheOncologist.com.

ABSTRACT

On March 16, 2007, eculizumab (Soliris®; Alexion Pharmaceuticals, Inc. Cheshire, CT), a humanized monoclonal antibody that binds to the human C5 complement protein, received accelerated approval by the U.S. Food and Drug Administration for the treatment of patients with paroxysmal nocturnal hemoglobinuria (PNH) to reduce hemolysis. Eculizumab was studied in a randomized, double-blind, placebo-controlled clinical trial in 87 RBC transfusion–dependent adult PNH patients and in a supportive single-arm study in 96 patients. The eculizumab dose was 600 mg as a 35-minute i.v. infusion administered weekly for the first 4 weeks followed by 900 mg (week 5) then 900 mg every 14 days thereafter. Hemoglobin stabilized in 21 of 43 (48.8%) eculizumab-treated patients, compared with none of 44 placebo-treated patients. Eculizumab-treated patients required significantly fewer RBC transfusions than placebo-treated patients (median, 0 versus 10 units). There was also a significant reduction in the serum lactate dehydrogenase area under the curve with eculizumab compared with placebo treatment. Results of the phase II supportive study were similar to those of the phase III study. The safety database included 196 adult patients with PNH. Significant findings included the development of human anti-human antibody responses in three patients and serious meningococcal infections in three patients. Patients should undergo meningococcal vaccination at least 2 weeks prior to receiving the first eculizumab treatment and have revaccination according to current medical guidelines. Patients must

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be monitored and evaluated immediately for early signs of meningococcal infections and treated with antibiotics as indicated. ***The Oncologist*** 2008;13: 993–1000

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is an uncommon, life-threatening hemolytic anemia. The disease is characterized by acute exacerbations of chronic intravascular hemolysis, venous thrombosis with predilection for intra-abdominal and cerebral veins, and bone marrow failure [1, 2]. Other common symptoms, likely related to hemoglobin-mediated nitric oxide scavenging, include smooth muscle dystonias of the gastrointestinal tract, manifested as dysphagia, esophageal spasm, and abdominal pain; vascular constriction, possibly causing erectile dysfunction and pulmonary hypertension; endothelial dysfunction, causing hypertension and increased vascular permeability and renal dysfunction with acute or chronic renal failure; and Fanconi syndrome (glycosuria, hyperaminoaciduria, hyperphosphaturia, and bicarbonate and water loss) [3, 4].

The cellular abnormality of PNH is caused by an acquired somatic mutation in the X chromosome of a totipotent hematopoietic stem cell [5]. This mutation leads to a deficiency in phosphatidylinositol glycan class A protein critical for the biosynthesis of glycosylphosphatidylinositol membrane anchoring proteins (GPI-APs). As a result, there is a partial (type II) or complete (type III) deficiency of GPI-APs on the surface of PNH stem cells. Two such deficient proteins are the complement inhibitors CD55 and CD59. Failure of complement regulation by these proteins renders PNH erythrocytes susceptible to complement-mediated lysis.

Diagnosis of PNH can be established by flow cytometric determination of the proportion of GPI-AP–deficient erythrocytes or granulocytes as determined using appropriate monoclonal antibodies and the FLAER assay (GPI-APs are receptors for pro*aer*olycin, a bacterial toxin that can be *fl*uoresceinated). The latter test enhances sensitivity and specificity for the diagnosis of PNH [6].

Small to moderate numbers of PNH clones are found in up to 70% of patients with acquired aplastic anemia, and GPI-AP–deficient cells have also been reported in patients with myelodysplastic syndrome (MDS). Occasionally GPI-AP–deficient cells are found in normal subjects but these are polyclonal and only manifest in committed cells with no self-renewal capability [7].

Prior to the approval of eculizumab, there was no approved therapy for PNH. Therapies commonly used included blood transfusion, erythrocyte-stimulating agent therapy, corticosteroids, anabolic steroids, oral iron therapy for iron deficiency, and bone marrow transplantation. For patients who developed venous thromboembolism, treatment included anticoagulation with low molecular weight heparin followed by chronic warfarin therapy [8].

Eculizumab (Soliris®; Alexion Pharmaceuticals, Inc. Cheshire, CT) is a humanized monoclonal antibody that binds to the human C5 complement protein with high affinity, thereby inhibiting its cleavage to C5a and C5b and preventing the generation of the terminal complement complex C5b-9 (membrane attack complex), responsible for the lysis of PNH RBCs lacking cell surface terminal complement inhibitor CD59 [9].

Biochemically, eculizumab is an IgGκ immunoglobulin comprised of human constant regions and murine complementarity determining regions grafted onto human framework light and heavy chain variable regions. Eculizumab is composed of two 448–amino acid heavy chains and two 214–amino acid light chains and has a molecular weight of approximately 148 kDa. Eculizumab does not inhibit C5 activity in animal sera but does bind to human tissues, including smooth and striated muscle as well as renal proximal epithelium. In animal studies, eculizumab was found to cross the placental barrier and show fetal morbidity and mortality [9].

The metabolism of eculizumab is understood to be via lysosomal enzymes to small peptides and amino acids, as is described for other proteins. In humans, the volume of distribution of eculizumab approximates that of plasma.

Pharmacodynamic assays demonstrate that inhibition of C5 sufficient to prevent hemolysis occurs at eculizumab plasma concentrations in excess of 35 μg/ml. Maximum plasma concentrations of eculizumab with therapeutic doses of the drug are achieved within 1 hour of infusion.

Patients and Methods

Submitted clinical data include a randomized double-blind, placebo-controlled, multicenter study of eculizumab or placebo administered i.v. to patients with PNH (the TRIUMPH study [A Hemoglobin Stabilization in Transfusion Reduction Efficacy and Safety Clinical Investigation, Randomized, Multicenter, Double-Blind, Placebo-Controlled, Using Eculizumab and Paroxysmal Nocturnal Hemoglobinuria Patients]) and a supporting open-label, phase III study (the SHEPHERD study [Safety in Hemolytic Paroxysmal Nocturnal Hemoglobinuria Patients Treated with

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Eculizumab: The Multicenter, Open Label, Research Design Study]) [10–12].

The primary objective of the TRIUMPH trial was to evaluate the safety and efficacy of eculizumab in transfusion-dependent patients with PNH. The trial consisted of a 2-week screening period, an observation period up to 3 months, and a 26-week treatment period. The coprimary efficacy endpoints were hemoglobin stabilization and the number of packed RBC units transfused during the treatment phase of the study. The secondary objectives of this study were transfusion avoidance and hemolysis as measured by lactate dehydrogenase (LDH) area under the curve (AUC) from baseline through week 26. This study also had exploratory endpoints of LDH change from baseline through week 26, health-related quality of life (QoL), as measured by the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire (EORTC QLQ)-C30 from baseline through week 26, as well as measures of thrombosis, platelet activity, nitric oxide, and free hemoglobin.

The primary objective of the SHEPHERD study was to evaluate the safety of eculizumab in patients with transfusion-dependent PNH. The secondary objectives of that study were to analyze hemolysis reduction as measured by LDH AUC and changes in the Functional Assessment of Chronic Illness Therapy–Fatigue (FACIT-F) QoL scale. Exploratory endpoints included health-related QoL as measured by the EORTC QLQ-C30 and measures of thrombosis, platelet activity, nitric oxide, and free hemoglobin.

The major inclusion criteria for the TRIUMPH trial were age $\geq$18 years, RBC transfusion requirement, CD59 cell clone $\geq$10%, stable doses of erythrocyte-stimulating agents, immunosuppressives, iron, folic acid, corticosteroids, and anticoagulants, and vaccination against *Neisseria meningitides* at least 14 days prior to treatment. In addition, patients were required to have an LDH level $\geq$1.5 times the upper limit of normal. Patients had to have had RBC transfusions if their hemoglobin was $\leq$9 g/dl with symptoms or if their hemoglobin was $\leq$7 g/dl without symptoms, and also within 1.5 g/dl of the mean hemoglobin pretransfusion value for the previous 12 months. A platelet count $\geq$100,000/μl and an absolute neutrophil count $\geq$500/μl were also required. Excluded patients included those with a history of bone marrow transplantation or meningococcal disease presence or suspicion of other bacterial infection, known or suspected hereditary complement deficiency, and pregnant or breast-feeding women or women intending to conceive during the course of the study, including the post-treatment phase.

Treatment assignment was stratified based on the number of packed RBC transfusions within 1 year prior to screening (low stratum, 4–14 units; middle stratum, 15–25 units; high stratum, >25 units). Patients randomized to the placebo group received placebo i.v. once a week for five doses then once every 2 weeks for 21 weeks. Patients randomized to the eculizumab group received eculizumab at a dose of 600 mg via a 35-minute i.v. infusion weekly for the first 4 weeks then 900 mg (week 5) then 900 mg every 14 days thereafter for 26 weeks total treatment. Following the 26 weeks of treatment, there was an 8- to 12-week post-treatment follow-up period. The study drug and placebo for the treatment period were supplied by Alexion Pharmaceuticals, Inc., Cheshire, CT.

Patients enrolled in the SHEPHERD study received the same eculizumab dosing regimen as used in the TRIUMPH study. This study had a 12-week enrollment phase followed by a 2-week screening phase followed by a 52-week treatment phase and an 8-week post-treatment phase. The inclusion and exclusion criteria were similar to those of the TRIUMPH study with the exception that patients in the SHEPHERD study were required to have one or more transfusions in 24 months, compared with four or more transfusions in 12 months in the TRIUMPH study, and a platelet count $\geq$30,000/μl, compared with a platelet count $\geq$100,000/μl.

For the primary analysis, the hemoglobin set point for each patient was defined as $\leq$9 g/dl in patients with symptoms and $\leq$7 g/dl in patients without symptoms. To achieve a designation of hemoglobin stabilization, a patient had to maintain a hemoglobin concentration above the hemoglobin set point and avoid any RBC transfusion for the entire 26-week treatment period. Patients who reached or dropped below their predetermined hemoglobin set point did not achieve hemoglobin stabilization. Those patients who withdrew prematurely from the study or were transfused above their hemoglobin set point during the treatment phase also were treated as not achieving hemoglobin stabilization. A two-sided Fisher's exact test was used for statistical analysis. Each patient's units of packed RBCs transfused after randomization through visit 18 were calculated. For those patients who discontinued the study drug but remained in the study for follow-up, the actual transfusion records were used to calculate the units transfused. For patients who had at least one transfusion but withdrew prematurely from the study prior to 26 weeks, the number of units was prorated by applying the formula (26/number of weeks on study drug) $\times$ (number of units transfused while on study drug). For patients who withdrew prematurely from the study prior to having a transfusion, their transfusion data for the 26 weeks previous to their last contact date was used to calculate the number of units of packed RBCs transfused. The primary analysis method was the Wilcoxon rank-sum test.

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Table 1. Demographics and baseline characteristics for patients in the TRIUMPH study

	Eculizumab (n = 43), n (%)	Placebo (n = 44), n (%)
Gender		
Male	20 (46.5)	15 (34.1)
Female	23 (53.5)	29 (65.9)
Age (years)		
Median	41.0	35.0
Range	20.0–85.0	18.0–78.0
Race		
White	37 (86.0)	41 (93.2)
Other	5 (11.6)	2 (4.6)
Not reported	1 (2.3)	1 (2.3)
Transfusion history		
n of units PRBCs transfused in prior 12 months		
Mean (g/dl) (standard deviation)	19.2 (8.41)	19.9 (9.28)
Median	18.0	17.0
Range	7.0–36.0	7.0–44.0
Symptoms associated with transfusion		
Change in mental status	14 (3.2)	20 (4.7)
Severe or worsening shortness of breath	69 (15.8)	63 (14.8)
Severe or worsening fatigue	279 (63.7)	264 (62.0)
Other	76 (17.4)	79 (18.5)
Thrombosis history, n (%)		
Any thrombosis event	9 (20.9)	8 (18.2)
Cerebrovascular accident	1 (2.3)	0 (0.0)
Mesenteric vein thrombosis	0 (0.0)	2 (4.5)
Thrombophlebitis/deep vein thrombosis	4 (9.3)	6 (13.6)
Other[a]	–	0 (0.0)
Concomitant antithrombotic agents, n (%)	24 (55.8)	20 (45.5)
Concomitant systemic corticosteroids, n (%)	13 (30.2)	15 (34.1)
Hemoglobin set value (g/dl), mean (standard deviation)	7.8 (0.79)	7.7 (0.75)
Disease duration (years), median (range)	4.3 (0.93–29.8)	9.3 (0.47–38.5)
Aplastic anemia, n (%)		
Yes	6 (14.0)	12 (27.3%)
No	37 (86.0%)	32 (72.7%)
Myelodysplastic syndrome, n (%)		
Yes	2 (4.7%)	0 (0.0%)
No	41 (95.3%)	44 (100%)
Baseline PNH type III RBCs (%)		
Mean (standard deviation)	28.0 (13.3)	35.1 (18.2)
Median	25.6	34.4
Range	2.4–54.0	6.6–88.0
Baseline free hemoglobin (mg/dl), median (range)	40.5 (7.5–764.0)	46.2 (11.2–502.0)
Baseline LDH (U/l), mean (standard deviation)	2,200 (1,034)	2,258 (1,027)
FACIT-F score,[b] mean (standard deviation)	36.7 (10.5)	34.3 (12.0)
Platelet count ($\times 10^9$/l), mean (standard deviation)	188.8 (83.3)	166.0 (94.2)
Hemoglobin (g/dl), mean (standard deviation)	10.0 (1.2)	9.7 (1.2)

[a]Other thrombosis included one event each of ischemic stroke, suspected pulmonary embolus during pregnancy, thrombus (not otherwise specified), thrombus artery carotis interna left, thrombus right arm after feeding infusion, pulmonary embolus, and transient ischemic attack.
[b]The FACIT-Fatigue tool was administered prior to transfusion.
Abbreviations: FACIT-F, Functional Assessment of Chronic Illness Therapy–Fatigue; LDH, lactate dehydrogenase; PNH, paroxysmal nocturnal hemoglobinuria; PRBCs, packed red blood cells; TRIUMPH, A Hemoglobin Stabilization in Transfusion Reduction Efficacy and Safety Clinical Investigation, Randomized, Multicenter, Double-Blind, Placebo-Controlled, Using Eculizumab and Paroxysmal Nocturnal Hemoglobinuria Patients.

Secondary efficacy analyses included transfusion avoidance, LDH AUC from baseline to week 26 and change in FACIT-F scale (version 4) score from baseline to week 26. Those patients who withdrew prematurely from the study during the treatment phase were considered as requiring transfusion. The analysis was carried out using the two-sided Fisher's exact test. The AUC of LDH from baseline through visit 18 was calculated for each patient. For patients with missing LDH values, the last-observation-carried-forward method was used to impute missing values. The AUC was analyzed using the Wilcoxon rank-sum test. The scoring guideline for the FACIT-F scale was used to

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Table 2. Analysis of primary efficacy endpoints

	Eculizumab, *n* (%)	Placebo, *n* (%)	*p*-value
Overall (*n*)			
Hemoglobin stabilization			<.0001
Yes	21/43 (48.8)	0/44 (0.0)	
No	22/43 (51.2)	44/44 (100)	
Median units PRBCs transfused	0.0	10.0	<.0001
4–14 units (*n*)			
Hemoglobin stabilization			<.0001
Yes	12/15 (80.0)	0/15 (0.0)	
No	3/15 (20.0)	15/15 (100)	
Median units PRBCs transfused	0.0	6.0	<.0001
15–25 units			
Hemoglobin stabilization			.0191
Yes	5/17 (29.4)	0/18 (0.0)	
No	12/17 (70.6)	18/18 (100)	
Median units PRBCs transfused	2.0	10.0	.0007
>25 units			
Hemoglobin stabilization			.0902
Yes	4/11 (36.4)	0/11 (0.0)	
No	7/11 (63.6)	11/11 (100)	
Median units PRBCs transfused	3.0	18.0	.0003

Abbreviation: PRBCs, packed red blood cells.

calculate the fatigue score. The main hypothesis of interest was that eculizumab would provide a statistically significant higher patients' total FACIT-F scale score than placebo. The change in the total FACIT-F score from baseline was analyzed using a mixed-effects model with baseline as covariate, treatment and time as fixed effects, and patient as a random effect.

Results

In the TRIUMPH study, 114 patients were screened, 108 entered the observation phase, and 88 protocol-eligible patients were randomized. One patient was removed from the analysis because of being randomized in error; therefore, 87 patients entered the treatment phase. Of the patients treated, 85 completed all visits. Two patients randomized to eculizumab discontinued treatment prematurely, one because of pregnancy and one because of not wanting to travel; 10 patients in the placebo group discontinued study drug prematurely because of a lack of efficacy but completed all study visits for follow-up.

The analysis population (intent-to-treat [ITT]) included all 87 randomized and treated patients. Demographics and baseline characteristics of the ITT population are shown in Table 1. There were no statistically significant differences between treatment groups in the pretreatment characteristics of patients.

Results of the primary efficacy analysis after 26 weeks on study are summarized in Table 2. Significantly more patients in the eculizumab treatment group showed hemoglobin stabilization than in the placebo treatment group (48.8% versus 0.0%; $p < .0001$), and patients in the eculizumab treatment group required significantly fewer packed RBC transfusions than patients in the placebo group (median, 0.0 units versus 10.0 units; $p < .0001$). For each of the three treatment strata, eculizumab treatment significantly decreased RBC transfusion requirements, and for the first two strata, eculizumab treatment significantly increased the percentage of patients with hemoglobin stabilization.

Results of secondary efficacy analyses for the overall study populations are shown in Table 3. Outcomes were statistically significantly better in the eculizumab treatment group than in the placebo group for each of these secondary endpoints. The favorable result for eculizumab with regard to efficacy was apparent across all randomization strata.

The study was not able to evaluate for efficacy of eculizumab with regard to prevention of thrombosis because most of the patients were receiving concomitant antithrombotic agents during the study and there was only one throm-

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Table 3. Efficacy analysis at completion of 26 weeks on study

Endpoint	Eculizumab (n = 43)	Placebo (n = 44)	p-value
Hemoglobin stabilization			
Yes	21/43 (48.8)	0/44 (0.0)	<.0001
No	22/43 (51.2)	44/44 (100)	
Units of PRBCs transfused			<.0001
Mean (standard error)	3.0 (0.67)	11.0 (0.83)	
Median	0.0	10.0	
Range	0.0–16.0	2.0–21.0	
Avoidance of transfusion			<.0001
Yes	22/43 (52.1%)	0/44 (0.0%)	
No	21/43 (48.8%)	44/44 (100%)	
LDH AUC (baseline to 26 weeks), U/l × day			<.0001
Mean (standard error)	81,140	459,116	
Median	58,587	441,880	
Range	32,417–792,005	234,604–711,934	
Change in FACIT-F score from baseline to week 26			<.0001
n	41	39	
Mean (standard error)	6.4 (1.2)	−4.0 (1.7)	
Median	4.0	−2.0	
Range	−5.0–28.0	−38.0–17.0	

Abbreviations: AUC, area under the curve; FACIT-F, Functional Assessment of Chronic Illness Therapy–Fatigue; LDH, lactate dehydrogenase; PRBCs, packed red blood cells.

botic event (hepatic vein thrombosis in a patient in the placebo treatment group) during the study.

Overall, 96 of the 97 enrolled patients completed the open-label supportive study (one patient died following a thrombotic event). A reduction in intravascular hemolysis as measured by serum LDH levels was sustained for the entire treatment period and resulted in a reduced need for RBC transfusions.

Patients were tested for human antihuman antibodies (HAHAs) after eculizumab treatment, and two patients were found to have IgG titers of 1:20 and one patient had IgM titers of 1:20 and 1:100. No patient developed a rebound in hemolysis with these antibodies. Also, because of concern of rebound hemolysis after discontinuing eculizumab, it was planned to follow patients after discontinuation of eculizumab, but few patients in the controlled study discontinued eculizumab prematurely and most patients continued into extension studies of eculizumab after completing study treatment.

Safety

Safety data were available from a total of 236 eculizumab-treated PNH patients including 43 patients from the

Table 4. Adverse reactions occurring in ≥5% of patients in the TRIUMPH study

Reaction	Eculizumab (n = 43), n (%)	Placebo (n = 44), n (%)
Headache	19 (44)	12 (27)
Nasopharyngitis	10 (23)	8 (18)
Back pain	8 (19)	4 (9)
Nausea	7 (16)	5 (11)
Fatigue	5 (12)	1 (2)
Cough	5 (12)	4 (9)
Herpes simplex infections	3 (7)	0
Sinusitis	3 (7)	0
Respiratory tract infection	3 (7)	1 (2)
Constipation	3 (7)	2 (5)
Myalgia	3 (7)	1 (2)
Pain in extremity	3 (7)	1 (2)
Influenza-like illness	2 (5)	1 (2)

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

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TRIUMPH study and 193 patients from uncontrolled PNH clinical studies. In the controlled study, two patients in the eculizumab group discontinued prematurely, one because of an adverse event (pregnancy, which went on to delivery of a normal infant) and one because of patient choice. The most frequent adverse events occurring during this study are summarized in Table 4. Adverse events that were notably more common in eculizumab-treated patients than in placebo-treated patients were headache, back pain, fatigue, and respiratory tract infection. Viral infection, urinary tract infection, and PNH were more frequent in the placebo group. No patients died.

Among the 193 PNH patients who received eculizumab in uncontrolled studies, serious adverse events occurring in two or more patients were viral infection (2.6%), headache (2.1%), anemia (1.6%), pyrexia (1.6%), and hemolysis (1.0%). Across all PNH studies overall, infections did not seem to be more frequent in patients who received eculizumab than in those who received placebo. The incidence of herpes simplex infection appeared to be greater in PNH patients treated with eculizumab (8 of 140; 5.7%), versus none in the pivotal trial placebo control.

Because eculizumab inhibits complement activation, it may impair neutrophil and monocyte function and impair the ability of the patient to clear infections with encapsulated organisms. There were three cases of *Neisseria* meningitis in patients receiving eculizumab, including a 22-year-old unvaccinated woman with idiopathic membranous glomerulonephritis who had received eculizumab for about 7 months, a 54-year-old vaccinated female PNH patient who had been on eculizumab for approximately 14 months, and a 24-year-old male vaccinated PNH patient who had received eculizumab for approximately 12 months. No patient died, but the unvaccinated patient had a complicated course with amputation of parts of some digits because of gangrene, pulmonary embolus, and pneumonia. As a result of this experience, all study patients were required to be vaccinated against *Neisseria meningitides* at least 2 weeks prior to starting study treatment.

A total of four eculizumab-treated PNH patients died. These include a 31-year-old man with PNH and hemosiderosis who suffered a pulmonary embolus and a hemorrhagic cerebral infarction 31 days after the last eculizumab dose; a female patient with cholecystitis who became septic and died from a cerebrovascular accident about 2 months after her last eculizumab dose; a man with MDS who developed cellulitis, sepsis, and acute renal failure after a fish hook infection and died from worsening of his MDS; and a woman who died of metastatic adenocarcinoma after 13 months on eculizumab.

Discussion

Eculizumab appears to be generally safe and effective for the treatment of PNH patients with transfusion-dependent hemolysis. The efficacy of eculizumab was demonstrated by the stabilization of hemoglobin and a lower number of packed RBC transfusions in these patients. Eculizumab also improves clinically measured hemolysis parameters such as LDH. The small number of patients, the low thrombotic event rate, and the high rate of concomitant antithrombotic agent use in the controlled clinical study preclude meaningful assessment of the effect of eculizumab on thrombosis in PNH patients. Measurement of QoL as assessed by the FACIT-F scale showed improvement in the eculizumab treatment group; however, the tool was administered to patients prior to transfusion without post-transfusion tool administration, so it is not possible to determine whether any difference between groups in the FACIT-F score is a result of the eculizumab or the anemia correction. Also, the FACIT-F scale has not been validated in PNH patients.

As part of the approval of eculizumab, the sponsor agreed to a number of postmarketing commitments. The sponsor will evaluate the long-term safety of eculizumab by analyzing outcomes in the PNH registry program for a period $\geq$5 years. The sponsor will also evaluate serious infections necessitating or prolonging hospitalization or resulting in death. The rate of malignancy, including the nature of the malignancy and the survival status of PNH patients who develop a malignancy, will be determined. For women who become pregnant, the sponsor will evaluate the clinical course and any congenital abnormalities among offspring. A randomized clinical trial to assess the effects of anticoagulant withdrawal among patients with PNH will also be initiated. The sponsor will also evaluate HAHA formation.

Patients with PNH who are treated with eculizumab are at a higher risk for infections with *Neisseria meningitides* [13]. An early alert program and a registry program have been instituted to minimize the risk for infections. Patients who are to be treated with eculizumab must be vaccinated with *Neisseria meningitides* vaccine at least 2 weeks prior to treatment with eculizumab.

Pregnant women with PNH and their fetuses have high rates of morbidity and mortality during pregnancy and the postpartum period. There are no adequate and well-controlled studies of eculizumab in pregnant women. It is likely that eculizumab will cross the placenta and appear in breast milk.

Eculizumab has not been studied in patients $\leq$18 years old. Experience with eculizumab in the geriatric population is limited; in PNH studies, only 15 patients aged $\geq$65 years

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were treated with eculizumab. Although there were no apparent age-related differences observed in these studies, the number of patients aged ≥65 is not sufficient to determine whether they respond differently from younger patients.

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The views expressed are the result of independent work and do not necessarily represent the views and findings of the U.S. Food and Drug Administration.

AUTHOR CONTRIBUTIONS

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Appeal T 1087/19-3.3.01 re. Opposition against European patent EP 2 359 834 (Application No. 11 001 632.6)
Title: Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement
Proprietor: Alexion Pharmaceuticals, Inc.
Opponent: Amgen Inc.
Our Ref.: AMG17203OP

In Response to the Statement of Grounds of Appeal filed by the Appellant on June 24, 2019

1. This is in response to the Statement of Grounds of Appeal filed by the Appellant and Proprietor on June 24, 2019 in respect of European Patent No. 2 359 834 (hereinafter "the opposed patent").

I. Requests

2. We request that the appeal be dismissed and the decision of the Opposition Division (hereinafter "OD") to revoke the patent in its entirety be maintained.

3. Oral Proceedings are requested in accordance with Art. 116 EPC in the event the Board of Appeal is of the opinion that our request to dismiss the appeal cannot be granted on the basis of our written submissions.

4. We further request that **D55** to **D60**, filed with the Appellant's submission dated June 24, 2019 not be admitted into the proceedings as they are late filed and not *prima facie* relevant for the case.

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II. Documents Referred to in the Present Submission

5. For the sake of procedural efficiency, we refrain from re-filing documents **D1** to **D32** and **D42** to **D53** relied upon by us or **O2** during opposition proceedings, however, we will gladly provide any of these documents should this be deemed expedient by the Board.

6. In addition, we herewith file additional documents **D61** and **D62**:

   D61 Anonymous TPOs filed for EP 3 167 888 on September 4, 2019.

   D62 Declaration of Tom Vink, filed in EP 1 558 648.

III. The Main Request

III.1 Added Matter (Art. 100 (c) EPC / Art. 76 (1) EPC, 123(2) EPC)

7. The OD erroneously considered the granted claim of the opposed patent to comply with Articles 123(2) and 76(1) EPC. As we have explained in detail in our Notice of Opposition dated August 4, 2017 (section V) and noted in our submission dated November 21, 2018 (section III thereof), the subject matter of the granted claim is not derivable in a direct and unambiguous manner from the parent or divisional applications as filed.

8. Briefly, the original application (**WO**, page 5, lines 3-9 and page 22, lines 7-14) refers to a 300 mg eculizumab single-use dosage form comprising 30 mL of a 10 mg eculizumab/mL sterile, preservative free solution only in the context of a single unit dosage form.

9. The two paragraphs of the application that teach a "single-use" dosage form in each case are prefaced by the teaching *"[i]n certain embodiments, said antibody that binds C5 or an active antibody fragment thereof is administered in a single unit dosage form"* (**WO**, p. 5, li. 3/4; p. 22, li. 7/8, emphasis added). The skilled person would thus generally understand the embodiments described in said passages to be "single-unit" dosage forms.

10. It is clear from our previously submitted considerations that the term "single-unit dosage form" would be interpreted by the skilled person as referring to a container *"designed to hold a quantity of drug product intended for administration as a single dose or a single finished device intended for use promptly after the container is opened"* (cf. **D10**, p. 2, 2nd par., emphasis added).

11. The referred to passages of the original disclosure thus indicate that the dosage forms disclosed therein contain an amount of the drug that is intended to be administered as a single dose, i.e., a single dose of 300 mg eculizumab.

12. We respectfully note that the OD erred in their assessment that the single-use dosage form recited in the granted claim was disclosed as a separate embodiment from the single-unit dosage form taught in the same passages. For clarification, page 5, lines 3 to 9 are cited below:

> *"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."* (**WO**, p. 5, li. 3 to 9, emphasis added)

13. As is obvious from the above, the relevant passage recites a single-unit dosage form no less than five times. This would leave the skilled person without any doubt that the passage as a whole, including the last sentence, is directed to single-unit dosage forms.

14. The granted claim being a medical use claim (cf. Proprietor's statement of grounds of appeal, section 4.4), the dose to be administered is undoubtedly a critical feature in the context of the claimed subject matter. However, the granted claim does not limit the dose of eculizumab in any way. The omission of the limitation to single doses (of 300 mg) results in an isolation of the feature 300 mg single-use dosage form from the more limited context in which it is disclosed in the earlier and divisional application as filed.

15. The claim as granted therefore violates Articles 123(2) and 76(1) EPC.

III.2 Priority (Article 87 EPC)

16. The OD confirmed our view that the granted claim does not enjoy the priority of US 2006/0783070 P (**P**). The priority document nowhere discloses a 300 mg eculizumab single-use dosage form comprising 30 ml of a 10 mg eculizumab/mL sterile, preservative free solution.

17. This has not been disputed by the Patentee.

18. The filing date of the opposed patent is thus the filing date of the corresponding international application, i.e., March 15, 2007.

III.3 Inventive Step (Article 100(a) EPC / Article 56 EPC)

*III.3.1 The distinguishing features over **D1***

19. The granted claim was refused by the OD on the basis that it did not comply with the provisions of Article 56 EPC (cf. p. 12, 7th par. of decision revoking the European Patent, hereinafter "the decision"). **D1** was considered to constitute the closest prior art, an assessment that has not been challenged by the Patentee.

20. The OD reasoned *inter alia* that in contrast to the position the Patentee has adopted during oral opposition proceedings, the differences between **D1** and the granted claim do not include the sequence of the claimed antibody. Instead, given that the granted claim does not specify a sequence of eculizumab and that the definition of the antibody in the specification is not exhaustive, it could not be determined with certainty, whether the eculizumab recited in the granted claim and the eculizumab disclosed in **D1** would actually be different from each other (p. 9, ultimate par. of decision revoking the patent). In essence, the OD thus found that the claimed antibody would be encompassed by the eculizumab disclosed in **D1**.

21. We agree with this assessment of the OD.

22. The Appellant alleges in this regard that the proper approach to claim interpretation was to take into account the meaning given to a term in the description (cf. statement of grounds of appeal, section 5.1.5). This is exactly the approach that has been adopted by the OD. The description of the opposed patent teaches that the antibody used in the method of inhibiting complement activity described therein was disclosed in US 6,355,245 (i.e., **D17**, cf. **B1**, [0054] and also **WO**, p. 16, li. 24) and was named eculizumab. The opposed patent thus explicitly teaches that eculizumab is disclosed in **D17**. The opposed patent further refers to Thomas *et al.* (**D13**), indicating that eculizumab was disclosed therein (cf. **B1**, par. [0003], reference 11).

23. Notably, **D1** cites the very same prior art documents, stating that they would disclose methods for the preparation of *"h5G1.1-mAb"*, that was *"currently undergoing clinical trials under the trade-name eculizumab"* (**D1**, [0052]).

24. Considering the references to identical documents in **D1** and the opposed patent, we fail to see that the Appellant convincingly demonstrated that the term "eculizumab" as used in the granted claim would be understood by the skilled person to define subject matter that is different from the "eculizumab" specified in **D1**.

25. It appears that, while the patent was – presumably with full intent – drafted based on a rather vague definition of the term "eculizumab", which of course potentially affords a wider range of protection, the Proprietor now finds itself in a situation where a narrower interpretation appears more suitable to its needs. This cannot, however, divert from the fact that Alexion has decided

to draft the patent specification in an ambiguous manner, instead of providing a clear and unambiguous definition of the term “eculizumab”.

26. When taking the content of the opposed patent as a whole into consideration, it becomes clear that the term “eculizumab” is not defined in an unambiguous manner to only encompass an antibody consisting of the amino acid sequences defined by SEQ ID NOs: 2 and 4. While par. [0103] of the opposed patent may be interpreted as indicating that “eculizumab” refers to an antibody comprising the amino acid sequences disclosed in this passage, further parts of the patent introduce ambiguity with regard to the definition of the term. This will be explained in further detail below.

27. As noted by the OD, **D17** does not explicitly disclose a full-length antibody to be eculizumab. The variable heavy and light chain sequences of eculizumab are disclosed in **D17** (**D17**, col. 44, 1st full par., SEQ ID NO: 20), together with a statement that the variable regions disclosed in **D17** may be combined with constant regions constructed of a mixture of constant domains from IgGs (**D17**, col. 45, 3rd full par.). By means of the reference to **D17**, the term eculizumab as used in the patent would thus encompass a range of antibodies with certain variable regions, which may be combined with a variety of different constant regions, in particular chimeric constant regions.

28. In addition to the definition of eculizumab by reference to **D17**, par. [0103] of the opposed patent teaches a set of sequences for one possible eculizumab heavy and light chain amino acid sequence, as noted above. The description, however, lacks a clear teaching that these SEQ ID NOs would define the only antibody encompassed by the term “eculizumab”, particularly in light of the alternative, broader definition taught by the patent by reference to **D17**.

29. While the specification does refer specifically to SEQ ID NOs 2 and 4, it does so only by describing them as “certain embodiments” of the antibody covered by the claim:

> *“The application provides a pharmaceutical composition comprising an antibody that binds C5 or an active antibody fragment thereof as characterised in the claim. The antibody that binds C5 or an active antibody fragment thereof is eculizumab.”* (**B1**, [0011], emphasis added);

see also

> *“Said antibody binds C5 and is eculizumab.”* (**B1**, [0016], emphasis added);

together with

> *“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. The pharmaceutical composition comprises eculizumab. 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."* (**B1**, [0018], emphasis added)

30. Paragraph [0011] of the opposed patent may be interpreted as indicating that the application as a whole relates to an antibody or to a group of antibodies that bind C5 and that this antibody or this group of antibodies (or fragments thereof) are termed eculizumab. Par. [0018] then may be seen as further specifying the C5 binding antibody to in *"certain embodiments"* comprise the heavy chain CDRs of SEQ ID NOs: 5, 6 and 7 and the light chain CDRs of SEQ ID NOs: 8, 9 and 10. Further exemplary embodiments of the C5 binding antibody that is eculizumab consist of SEQ ID NO: 2 and SEQ ID NO: 4.

31. Accordingly, these passages could be read as indicating that, while the claimed composition generally comprises an antibody termed "eculizumab" (which is a C5-binding antibody), only certain embodiments (i.e., not all embodiments) falling thereunder consist of SEQ ID NOs 2 and 4. Others may, e.g., only comprise the CDR regions of SEQ ID NOs: 5 to 10, or the variable heavy and light chain sequences disclosed in SEQ ID NOs 1 and 3. The Appellant has therefore drafted the specification in an ambiguous way, rendering it unclear whether the term "eculizumab" refers to one specific antibody, or to a group of antibodies sharing certain sequence features.

32. The description of the patent thus does not unambiguously limit the term "eculizumab" to only one specific antibody. This allegation by the Appellant is simply incorrect.

33. When it comes to the interpretation of not clearly defined terms in patent claims, the well-established principle is to assign to them the broadest technically sensible meaning. While the claims should be construed by a mind willing to understand, this does not mean that a broad term may be assigned a meaning that is narrower than what is defined by the patent application itself or dictated by common general knowledge. The Boards of Appeal have explicitly made clear that a "mind willing to understand" only requires ruling out claim interpretations that are illogical or do not make technical sense and that a broad term should be understood to include all technically logical meanings (**T 1584/08**, reasons 16, **T 1408/04**, reasons 1). When limiting a claim to specific embodiments, the limitation must be restricted to what is unambiguously disclosed to a skilled person taking into account the skilled person's

general knowledge (**T 1408/04**, reasons 1). Of course, the disclosure of a patent (application) must be considered as a whole, instead of only considering isolated sections. This, however, is exactly what the Appellant did when referring to par. [0103] of the patent in isolation, disregarding the passages discussed above.

34. The skilled person is thus confronted with an ambiguous definition of the term "eculizumab" in the specification of the opposed patent. Since the Appellant itself failed to set forth a clear meaning for the term "eculizumab", it cannot rely on a narrow definition to render the claimed subject matter inventive. The OD did not err in interpreting the term in a manner that reflects the vague definition assigned to it in the patent specification. Accordingly, the feature "eculizumab" cannot serve as a distinguishing feature over **D1**.

35. We further fully agree with the OD in that the sterility of the composition is implicit in **D1**, as the closest prior art teaches intravenous injection of an eculizumab formulation. This means that the distinguishing features (as defined by us and the OD) are

- the single-use dosage form of 300 mg
- in a concentration of 10 mg eculizumab/mL
- resulting in a total volume of 30 mL
- wherein the solution is preservative free.

36. The OD has further noted that the amount of 300 mg and the lack of preservative are already suggested by **D1**. This is in agreement with our arguments presented during opposition proceedings, which we refer to in this regard (Par. 25 to 28 and 42 of our notice of opposition, par. 5 to 7 of our submission dated November 21, 2018). The overall volume is obviously determined by the amount and concentration of the antibody and therefore does not constitute an independent feature. This leaves the concentration of 10 mg/mL as the only relevant distinguishing feature over the closest prior art document.

37. No specific technical effect arises from this distinguishing feature, as will be explained below.

38. If anything, 10 mg/mL represents one out of various suitable concentrations for a composition such as the claimed one.

39. If the concentration is to be used as a basis to formulate a technical problem, the skilled person starting from **D1** would be confronted with identifying a suitable antibody concentration that offers sufficient/acceptable stability for the resulting composition to be used in the treatment of PNH.

III.3.2 *The reformulation of the technical problem suggested by the Appellant is not allowable*

40. As we have explained above, the antibody recited in claim 1 of the Main Request cannot serve as a distinguishing feature over **D1**. However, should the Board agree with the Appellant and consider that "eculizumab" as recited in claim 1 may serve as a distinguishing feature over **D1**, the reformulation of the technical problem suggested by the Appellant should nevertheless be rejected.

41. If a problem to be solved is given in the original description of a patent application, the reformulation of the problem to be solved may be appropriate, if the new problem can be deduced from the application as filed. An effect provided by the invention may be used to reformulate the problem to be solved, as long as that effect is derivable from the application as filed, or if it can be deduced by the skilled person from the application in the light of the prior art. An inventive step may be based on new effects, submitted during examination proceedings, if these effects were implied by or related to the technical problem initially suggested, however, it is not permissible to change the nature of the invention (Case Law of the Boards of Appeal, 9th Ed. (hereinafter CLBA), I.D.4.4).

42. As we have explained in our previous submissions (cf. section IV.3 of our submission dated November 21, 2018), the problem to be solved is formulated in the original application as follows:

> *"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."* (**WO**, p. li. 24 to 28)

This is supported by various similar statements throughout the original disclosure. In accordance with this interpretation, the Proprietor formulated the underlying problem of the original filing to lie in the provision of a useful treatment for patients suffering from PNH in its written submissions during opposition proceedings (see, e.g., section 5.10.2 of the submission dated March 22, 2018).

43. As noted above, the parent application as filed also teaches that the

> *"antibodies disclosed in U.S. Patent 6,355,245* [i.e., **D17**] *include a preferred whole antibody (now named eculizumab)."* (**WO**, p. 16, li. 23/24)

44. The original specification refers to **D17** in a further passage that is introduced by the phrase *"Suitable anti-C5 antibodies are known to those of skill in the art"*, i.e., suitable antibodies to be used in the method of treatment provided by the application are known to the skilled artisan.

45. Accordingly, the parent application as filed is directed at the provision of methods of treatment for PNH using known antibodies, such as eculizumab.

46. Considering the original disclosure, which explicitly teaches that eculizumab has been described previously, a reformulation of the technical problem towards the provision of a new, i.e., previously unknown, antibody would not only not be derivable from the original disclosure, it would directly contradict it.

47. Nevertheless, the Appellant now tries to switch the analysis of inventive step towards the provision of a new antibody:

> *"The objective technical problem which is solved by the invention is therefore the development of an antibody for use in the treatment of PNH, in a composition which is highly stable, and which can be provided in a low volume liquid formulation."* (section 5.2.12 of statement of grounds of appeal)

48. The newly formulated problem to be solved contains several aspects which are neither originally disclosed nor implied by the original application:

- the development of an antibody (i.e., the provision of an (alternative) antibody),
- in a composition that is highly stable (i.e., maximizing stability of the antibody in solution upon storage), and
- the provision of a low volume liquid formulation.

49. Thus, the Patentee tries at this very late stage to reformulate the technical problem in a highly specific way that is unrelated to the original disclosure and is based on alleged technical effects of the claimed subject matter that are not hinted at, let alone disclosed in the parent application as filed.

50. On a side note, it is clear from the above, that, even if the problem formulated by the Appellant were accepted, it amounts to an aggregation of partial problems that relate to different alleged technical effects (if there are any at all).

III.3.2.1. The provision of an allegedly new antibody

51. As explained above, the development of an antibody, i.e., the provision of a new antibody is not a technical problem that may be derived from the parent application as filed. In fact, the teaching of the original specification would have to be disregarded in essential parts to arrive at a technical problem partially consisting of the provision of a formerly unknown antibody.

52. While it may generally be permissible in certain cases to reformulate the technical problem, the Boards of Appeal have made clear that such reformulation cannot lead to a technical problem which is in contradiction to the original teaching of the application as filed (cf.

T 2245/10, reasons 2.3.5). The application as filed explicitly teaches that eculizumab was known, i.e., that the problem to be solved lies in the provision of methods of treatment using known antibodies (cf. *supra*). A reformulation of the technical problem towards the provision of a new antibody (which is eculizumab) would thus clearly contradict the original teaching and hence cannot be allowed.

53. It should be noted that the Appellant was aware of **D1** as the closest prior art document at least as of July 2011. The very same document had already been cited as closest prior art by the Examiner during prosecution of the patent (cf. ESOP of July 21, 2011). **D1** and the opposed patent further name the same two inventors, Leonard Bell and Russell P. Rother. The content of **D1** was thus undoubtedly known to the Patentee when filing the application that gave rise to the opposed patent.

54. Moreover, the content of **D1** is highly similar to the content of **D3**, which is expressly referred to in the original filing (**WO**, p. 42, ref. 12). Referencing **D3**, the original specification states the following:

> *"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."* (**WO**, p. 1, ultimate par.)

55. This passage is followed immediately by the summary of the invention. The parent application as filed thus essentially refers to **D3** as the closest prior art. **D1** and **D3** relate to the very same clinical study, involving 11 patients suffering from PNH and contain a technical teaching which is highly similar in essential parts. According to the **WO**, the deficits of **D3** are: (a) the study was unblinded, (b) the number of patients was small and (c) the study did not involve a control arm.

56. It is nowhere mentioned that **D3** would fail to provide the antibody that is eculizumab, or that an (inferior) alternative antibody had been used in **D3**.

57. It should be kept in mind that the opportunity to reformulate a technical problem in a way that deviates from the technical problem as defined in the application as filed applies in particular in situations where the Applicant is faced with new prior art (CLBA, p. 192, penultimate par.). This is not the case here. The Appellant was aware of **D1** as of July 21, 2011, and, more importantly, was aware (from **D3**) of the essential aspects of the teaching of **D1** when drafting the patent application that formed the basis of the opposed patent! Nevertheless, the original specification nowhere refers to the provision of a new antibody. Quite to the contrary, it teaches that specifically eculizumab was known!

58. It is further notable that the Proprietor in multiple instances has defined the differences between the claimed subject matter of the granted claim and the closest prior art document **D1** during opposition proceedings (cf. submission dated March 22, 2018, section 5.2.1 and submission dated November 21, 2018, section 3.4) prior to oral opposition proceedings. In none of these cases did the Appellant refer to the amino acid sequence of eculizumab as being a feature distinguishing the claimed subject matter from the closest prior art.

59. The Patentee thus had ample time to consider possible distinguishing features in view of **D1** that could be taken into account for acknowledging an inventive step. Still, the Patentee decided to withhold the argument that the sequence of eculizumab was unknown until the day of oral opposition proceedings.

60. Importantly, it is unclear what technical problem would be solved by the allegedly new antibody sequence. When discussing the newly formulated technical problem, the Patentee mentions stability upon storage and the formulation being low-volume. How is this connected to the antibody sequence? The Appellant's statements do not provide any insights in this regard.

61. In section 5.5 of the statement of grounds, the patent Proprietor explains why the solution to the purported technical problem allegedly lies in *"the provision of eculizumab […]"* (par. 5.5.1). This appears to indicate that the technical problem as formulated by the Patentee is solved by the specific sequences disclosed in SEQ ID NOs: 2 and 4 of the opposed patent. This allegation lacks any factual basis.

62. There is no data on file, or in the patent application that would provide evidence, or merely a hint that the amino acid sequences defined by SEQ ID NOs: 2 and 4 would have any impact whatsoever on the (long-term) stability of the claimed formulation.

63. According to the Patentee, **D1**, in combination with **D17** and **D13**, which are cited therein would lead the skilled person to assume that "eculizumab" would be an antibody comprising IgG4 constant regions (the variable regions of SEQ ID NOs: 2 and 4 are disclosed in **D17**) (cf. par. 5.5.7 of statement of grounds). Following this logic, the difference over the prior art **D1** (together with the referenced documents **D13** and **D17**) would be the different constant region. How does this alternative constant region influence the aggregation tendency of the antibody? Again, the Patentee's statement of grounds provides no insights.

64. With the statement of grounds, the Appellant has filed **D57**, a declaration of Leonard Bell. This document relates to purported amino acid sequence differences between the antibody disclosed in **D17** and **D13** and the antibody marketed as eculizumab (which is not the antibody defined by SEQ ID NOs: 2 and 4). The Bell declaration also offers no explanation why the alleged structural differences between the antibody that is sold as eculizumab and the antibody that is disclosed in **D17** and **D13** would have any impact on the stability of the antibody during storage.

65. It is a well-established principle that features which do not contribute to solving the technical problem, either independently, or in conjunction with the other features recited in the claim should be disregarded when assessing inventive step (CLBA, I.D.9.5). Thus, in the context of the technical problem of providing a highly stable (low-volume) formulation, the allegedly distinguishing feature of the amino acid sequence has no technical effect and should therefore be disregarded for the assessment of inventive step.

66. In the case of a mere juxtaposition or aggregation of features that are functionally independent, i.e., do not mutually influence each other to achieve a technical success over and above the sum of their respective individual effects, partial problems exist (CLBA, I.D.9.2.2). This would be the case, if the assessment of inventive step was (partially) based on the allegedly new antibody sequence. The Appellant has not provided any arguments why and how the amino acid sequence of SEQ ID NOs: 2 and 4 would be functionally interconnected with the alleged stability of the antibody against aggregation.

67. When (once again) reformulating the technical problem, the Appellant suggested that part of the problem to be solved would lie in the *"development of an antibody for use in the treatment of PNH"* (par. 5.2.12 of the statement of grounds). At the same time, the Appellant explains that **D1**, in combination with **D13** and **D17** actually discloses an antibody that would by the skilled person be regarded as being eculizumab (par. 5.5.7 of the statement of grounds). Of course, **D1** also discloses that the antibody referred to therein as eculizumab is useful for the treatment of PNH.

68. Accordingly, **D1** (together with the documents cited therein) does provide an antibody useful in the treatment of PNH, even if the Board is minded to follow the Appellant's arguments. The technical problem therefore in any case cannot be the *"development of an antibody for use in the treatment of PNH"*. Such an antibody is already provided by the prior art. If anything, the technical problem arising from the distinguishing feature of SEQ ID NOs 2 and 4 could lie in the provision of an alternative antibody for the treatment of PNH.

69. This would be an entirely new (partial) problem, which is unrelated to the problem disclosed in the original teaching and unrelated to the technical problem suggested by the Appellant during written opposition proceedings. It is furthermore introduced extremely late by the Appellant despite the fact that the relevant facts were known to the Patentee before the filing date of the international application (cf. above).

70. It should be noted that the Bell declaration (**D57**) is dated September 9, 2016 and was filed by the Appellant in corresponding US patent proceedings. This irrefutably shows that the Appellant was aware of the alleged differences between the antibody sequences disclosed in **D13** and the opposed patent at least as of September 2016. In par. 2 of **D57**, Dr. Bell alleges that eculizumab had not been available to the public before the filing date of the opposed patent (March 15, 2007).

71. Nevertheless, the Patentee has (after the date of **D57**) made submissions during opposition proceedings before the EPO dated March 22, 2018 (par. 5.2.1) and November 21, 2018 (par. 3.4), wherein the allegedly distinguishing features over **D1** did not include the antibody sequence. The Appellant thus actively decided not to mention the allegedly distinguishing feature of the antibody sequence and withhold the argument until the day of oral opposition proceedings.

72. The Appellant thus tries to modify the technical problem formulated during opposition proceedings by resorting in an abusive manner to an allegedly distinguishing feature that is technically unrelated to the proposed technical effect suggested by the Patentee (low aggregation tendency/low formulation volume).

73. Due to the above reasons, a reformulation of the technical problem as suggested by the Appellant, i.e., to include the provision of a formerly unknown antibody (that is eculizumab) should not be allowed by the Board.

III.3.2.2. The formulation of a technical problem relating to galenic considerations

74. Regarding the second aspect of the technical problem as formulated by the Appellant, the provision of a low-volume, liquid formulation that is highly stable is a technical problem that relates to galenic questions. The opposed patent is, however, not concerned with galenic questions at all (cf. par. 76 to 89 of our submission dated November 21, 2018).

75. As may be taken from the Case Law of the Boards of Appeal (cf. above), the technical problem may solely be reformulated or clarified provided that the new problem can be deduced from the application as filed and the nature of the invention is not changed. Unsurprisingly, the Appellant refers to the Guidelines for Examination (G-VII, 11) to support its case. Therein, an example is given for an allowable reformulation of the problem to be solved, based on new evidence filed by the Applicant. For an invention relating to a pharmaceutical composition having a specific activity, a reformulation of the technical problem to include an unexpectedly low toxicity is considered allowable, since the skilled person allegedly always contemplates pharmacological activity and toxicity together.

76. This example is not applicable to the current case. Even if it is assumed, *arguendo*, that a skilled person considering the pharmacological activity of an active agent would indeed always consider the toxicity of said agent, there is no analogous consideration applying to the stability of an active agent. Dosing regimen are routinely adapted by medical practitioners in order to establish an equilibrium between the desired activity of an active agent and unwanted toxicity. However, even this would in general only be the case where toxicity is a limiting factor, which is not the case for each and every active agent. Therefore, this consideration does not generally apply to any type of active agent, in our view. We do not consider the logic formulated in the Guidelines G-VII, 11 to be generally applicable.

77. An obvious difference between the toxicity of a given pharmaceutical composition and aggregation of an active agent comprised therein is that toxicity represents a direct effect on the patient. Both pharmacological activity and toxicity are effects that directly relate to the well-being of the patient. This is not the case for the aggregate level of an antibody formulation. The level of antibody aggregates that form in such a formulation is a property of the composition itself that may (or may not) result in an effect on the patient. This, however, has not been shown for the claimed composition.

78. In any case, the Appellant has failed to provide any evidence which would show that a skilled person would always contemplate pharmacological activity and stability of an antibody active agent together. This is not the case. Antibody active agents are marketed with a certain predetermined expiration date. They are considered by the medical practitioner to be suitable for administration before that date and unsuitable after this date. No evidence was provided which would show that the skilled person would, e.g., adapt the dosing of an antibody composition in relation to its storage duration to compensate for loss of activity. No evidence was provided either that there is a clinically relevant (!) difference between an eculizumab solution with a monomer content of, e.g., 99.7% and a solution with a monomer content of, e.g., 98.6% (cf. **D33**, Fig. 2).

79. In light of the originally technical problem – the provision of a method of treatment for PNH – evidence needs to be provided that the purported technical effect has any actual relevance in a clinical situation, i.e., any relevance to the patient.

80. The Appellant tries to blur the lines between – on the one hand – the requirement of an antibody formulation being safe and effective, or suitable for administration and – on the other hand – maximizing the long-term stability of an antibody during storage. These are obviously distinct problems, the latter of which is in no way derivable from the parent application as filed.

81. In par. 5.3.6 of the statement of grounds, the Appellant states that it would be implicit from the application documents that the compositions described therein should are *"intended to be stored before use"* and that they should be *"safe and effective"*. In subsequent par. 5.3.9, the Appellant alleges that *"the need for highly stable formulations"* (emphasis added) would be clear from the application as filed from the need for storage of compositions and kits prior to their use in therapy and from the examples of "suitable formulations" (plural), which were developed to provide improved stability. This passage is highly misleading.

82. First of all, if the Appellant wishes to derive a need for highly stable formulations from the application documents, it should at the very least be expected that the relevance of a high antibody stability is mentioned somewhere in the application. This is not the case. The application as filed does not refer in any way to the relevance, let alone to an improvement of antibody stability. Even if it were assumed that the application as filed would indicate to the skilled person that the compositions disclosed therein should meet a specific stability

requirement (which is not the case), this could only be the requirement of a sufficient stability, as will be explained in more detail below.

83. Secondly, the Appellant argues that a need for storage of the compositions disclosed in the original teaching is derivable therefrom. However, during opposition proceedings, the Patentee has essentially argued about long-term stability. Obviously, a general need for sufficient storage stability for a composition to be useful as a drug product and the need for optimal long-term stability are two distinct requirements that are not freely exchangeable. Even if a need for a certain storage stability would be derivable from the application as filed (which we disagree with), this is not true with regard to optimal long-term stability.

84. As the skilled person knows, many pharmaceutical compositions are marketed that cannot be stored for long periods of time. In some cases, pharmaceutical formulations need to be freshly prepared and administered to the patient within only a few days. Nevertheless, active agents with limited stability, resulting in compositions which can be stored only for very short periods of time, are routinely used in, e.g., cancer therapy. Maximizing long-term stability is thus not a problem that is directly related to the medical treatment itself, but rather to economical or practical considerations, i.e., in the context of the provision or improvement of a commercial formulation.

85. By no means may long-term stability be regarded as a problem which the skilled person would always contemplate together with pharmaceutical activity. While there may be an economic interest in minimizing the amount of drug product that has to be discarded instead of being administered to a patient, this is not an issue that would be derivable from, or connected in any way to the contents of the original disclosure. This assessment is in full agreement with the position of the OD (p. 12, 2nd par. of the decision).

86. Thirdly, we note that the Applicant portrays the disclosure of the original filing in a highly misleading way. The application does not teach *"suitable formulations"* (plural), which were developed to provide *"improved stability"* (cf. statement of grounds, par. 5.3.9). Aside from very general statements, the application as filed comprises only a reference to one specific formulation having a defined concentration, which is 10 mg/mL (**WO**, p. 22, li. 11 to 14), but even this formulation is not clearly defined in terms of buffer, pH, excipients, etc. There is no disclosure of a plurality of *"suitable formulations"* in the original filing. The Appellant tries to generate the impression that the application as filed would contain a detailed teaching with regard to formulating the active agent eculizumab. This is not the case.

87. Notably, the examples of the application do not teach any formulation at all, let alone several formulations. It is not clear from the clinical study that is described in the examples of the original filing exactly which formulation was used therein. It is perfectly conceivable that a different formulation was used than the one recited in the granted claim. Furthermore, the examples do not contain any statement that would relate to the stability of the (undefined)

formulation used therein. Of course, this also means that there is no teaching of "improved stability" to be found in the examples, or, in fact, anywhere in the original teaching.

88. Accordingly, the international filing nowhere discloses that the formulation vaguely defined therein was *"developed in order to provide improved stability"*. Maybe the disclosed formulation was developed for improved stability. Maybe it was the first formulation that offered sufficient stability to market the drug as soon as possible. Maybe the concentration is the result of economic considerations that are unrelated to the subject matter of the parent application as filed, or are related to the production process of the antibody active agent. Maybe the concentration of 10 mg/mL has been picked from a range of concentrations that all proved to offer sufficient stability because it was convenient in some regard. All this is conceivable, because the original filing is completely silent in this regard. The Appellant's statements should be taken for what they are – mere allegations that have no basis in the original disclosure.

89. Even if it were assumed that the problem as formulated in the original description would imply a certain stability requirement, which we contest (cf. supra), such requirement could at best be that the provided formulation is sufficiently stable to be used in the disclosed treatment. The Appellant's argument that "sufficiently stable" would require the monomer content to be no less than 98% (cf. e.g., **D33**, par. 7) should be disregarded by the Board. This threshold is neither taught in the application as filed, nor part of the common general knowledge. There is no evidence on file that the skilled person would consider 98% monomer content to be the threshold below which an antibody formulation would be considered to be unsuitable for administration.

90. Quite to the contrary, we have filed **D20**, as evidence for common general knowledge of the skilled person in the relevant field and this document (a review article) indicates that, according to WHO standards, commercial intravenous immunoglobulin products (such as the composition referred to in the granted claim) are considered to be suitable for administration as long as they exhibit an aggregate level of less than 5% (**D20**, p. 9, left column, 1st paragraph). This means that, according to the WHO, any antibody solution comprising at least 95% antibody monomer would be suitable for intravenous delivery and could thus be released into the market.

91. Importantly, this means that, according to common general knowledge, all of the tested concentrations (**D23**: 2, 5, 10, 30 and 50 mg/mL, **D33** and **D55**: 10 and 30 mg/mL) in any of the tested buffers (**D55**) would be considered to be suitable for intravenous administration at all of the tested time points.

92. When a Patentee resorts to post-filed evidence to support an inventive step, the burden of proof generally lies with the Patentee (see e.g. **T 1188/00**, point 4.5 of the reasons, **T 1417/05**, point 9.4 of the reasons, **T 1034/14**, point 5.4 of the reasons, **T 235/04** point 4.6 to 4.8 of the

reasons). This applies to the question of whether the technical problem has been solved, whether it has been solved over the whole scope claimed and it should similarly apply to the question if the invoked effect is even related to the technical problem formulated in the application as filed. As a general rule, the definition of an artificial technical problem should be avoided (Case Law of the Boards of Appeal, 9th Ed., p. 191, 2nd par.).

93. In this regard, the Appellant has only provided allegations that are not in line with common general knowledge, which is evident from **D20**. The purported advantages in terms of antibody stability are not relevant in the context of the technical problem that is formulated in the original application. The threshold value of 98%, invoked by the Appellant is completely artificial and should not be taken into consideration when reformulating the technical problem.

94. It is important to note that, even if the claimed concentration of 10 mg/mL would actually provide a benefit with regard to long-term stability of the claimed (group of) antibodies, which it does not, particularly not over the whole scope claimed, these effects cannot be taken into consideration for an assessment of inventive step, because they are not hinted at in any way in the parent application as filed.

95. The Appellant relies on the Guidelines of Examination. As discussed above, however, the concept relied upon in the Guidelines must not be extended to any effect that may potentially be of benefit to a patient. In **T 845/02**, the claims in question related to an intrastromal corneal insert, i.e., a small device implanted into the eye to correct vision. The technical effects invoked by the Applicant in support of an inventive step included the avoidance of problems relating to corneal erosion and wound healing at the incision point. Such effects are obviously beneficial to a patient receiving a corneal implant such as the claimed one in **T 845/02**. This does not mean, however, that the Applicant may subsequently resort to such effects, despite them not being mentioned in the original documents:

> *"The other effects presented by the appellant as the avoidance of the problems of corneal erosion and wound healing have no basis nor justification in the application as filed. Therefore, contrary to the appellant's assertion, these alleged effects cannot be taken into account when determining the problem underlying the invention for the purpose of assessing inventive step (T 0386/89, 23 March 1992)."* (**T 845/02**, reasons 2.3, emphasis added)

96. The competent Board rightly refused the Appellants arguments in the case under appeal, because a too generous application of the (in any case questionable) logic laid out in section G-VII, 11 of the Guidelines for Examination would lead to a situation in which the Applicants of patent applications directed to medical devices or medical uses could at any time point draw from a vast reservoir of technical effects that may be of benefit to a patient, even if those effects are not mentioned at all in the original document. Such a situation would be at odds

with the principles formulated by the Boards of Appeal with regard to a reformulation of a technical problem (cf. CLBA, I.D.4.4.2), as discussed above.

III.3.2.3. Summary

97. In summary, the reformulation of the technical problem submitted by the Appellant should be refused by the Board, as is contains several elements that violate the applicable principles derivable from the relevant case law. The provision of a new/alternative antibody is a technical problem that is not only not derivable from the earlier application as filed, it even contradicts it. The provision of a highly stable, low volume composition, on the other hand, relates to galenic questions, which are not the subject of the original documents, not contemplated by the skilled person in the context of the originally formulated technical problem and not connected to any effect mentioned in the application as filed.

III.3.3 The problem put forward by the Appellant has not been credibly solved, in particular not over the whole scope claimed

98. In our previous submissions, we provided arguments why the technical problem of providing a highly stable formulation of eculizumab has not been credibly solved, let alone over the whole scope claimed (sections IV.3 and IV.4 of our submission dated November 21, 2018). These arguments are still valid.

99. In response, the Appellant has filed two further declarations of Dr. Dino Miano, i.e., **D54** and **D55**.

100. These declarations are insufficient evidence that the problem has been solved, or that it has been solved over the whole scope claimed.

101. It may be assumed that the studies executed by Dr. Miano (**D33**, **D54** and **D55**) have all been performed using the antibody which is commercially available under the tradename Soliris®. However, the opposed patent does not define the term "eculizumab" as being an antibody of this sequence. Even if the Board is minded to agree with the Appellant that "eculizumab" as used in the granted claim unambiguously refers to an antibody consisting of the amino acid sequences defined in SEQ ID NOs: 2 and 4, this is not the antibody that has been used for the experiments performed by Dr. Miano, because the commercial antibody lacks the leader peptide that is part of the light chain amino acid sequence defined by SEQ ID NO: 4 of the opposed patent.

102. It has not been demonstrated by the Appellant that an antibody defined by SEQ ID NOs: 2 and 4 of the opposed patent is sufficiently stable, let alone highly stable, when formulated at a

concentration of 10 mg/mL. Thus, the problem as defined by the Appellant cannot be regarded as being credibly solved by the subject matter of the Main Request.

103. As we have argued in our previous submissions, the property of the claimed composition exhibiting a low degree of aggregation is of no direct relevance in the context of the original disclosure. Biological activity of an antibody composition would appear to be much more relevant for the patient receiving the composition. In order to establish a causal link between aggregation and biological activity, the Appellant has submitted **D54**.

104. It is notable that **D54** does not contain any comparative data which would show that a solution of eculizumab at a concentration of 10 mg/mL exhibits a higher degree of biological activity than a solution of eculizumab at 30 mg/mL after storage. As described in **D54**, Dr. Miano has isolated eculizumab dimers (i.e., a particular type of aggregates) presumably from a sample of the commercial composition (**D54**, par. 3). Of course, dimers are not the only type of antibody aggregate, let alone the only type of degradation product that may form upon storage. These isolated dimers were then subjected to a C5 binding assay (**D54**, par. 4 and 7) and compared to a sample of the source material (although **D54** is not quite clear in this regard).

105. Dr. Miano has surprisingly not performed a direct comparison between two solutions (10 mg/mL and 30 mg/mL) after a certain period of storage. Given that many different degradation pathways contribute to a potential loss of biological activity, this is a significant omission. Based on **D54**, it is impossible to assess with certainty, or even with a reasonable degree of confidence, whether a 10 mg/mL solution of eculizumab would indeed possess a higher degree of biological activity than a 30 mg/mL solution. The alleged higher degree of aggregate formation in the 30 mg/mL solution may be compensated, and even overcompensated by, e.g., a lower degree of acidic variant formation.

106. It is still unclear, whether aggregation is the predominant degradation pathway for eculizumab. It is thus easily conceivable that solutions of eculizumab at alternative concentrations exhibit a higher biologic activity after prolonged storage, because degradative pathways other than aggregation are reduced in comparison to 10 mg/mL.

107. Notably, **D25** provides a direct comparison between solutions of eculizumab at different concentrations after storage over various amounts of time (cf. par. 39 to 43 of our submission dated November 21, 2018; **D25**, table 6, par. [0306]) and teaches that there is no significant difference. Due to the lack of such a direct comparison, **D54** cannot disprove the teaching of **D25**.

108. **D54** hence fails to provide evidence that the concentration of 10 mg/mL provides an advantage over other concentrations with regard to biologic activity of eculizumab.

109. **D55** comprises data supposedly showing that the concentration of 10 mg/mL provides superior stability regardless of the solution pH and formulation excipients.

110. We firstly note that there is a surprising degree of variation with regard to the amount of aggregate formed in the assays performed by Dr. Miano in **D33** and **D55**. In **D33** (cf. Fig. 1) an increase of the amount of aggregate of a tested 30 mg/mL solution of eculizumab from about 0.25% to about 1.4% (monomer content was reduced from about 99.75% to about 98.6%) within three months was considered to be representative of insufficient stability.

111. In **D55**, a similar assay was performed using 10 mg/mL solutions in different buffers. Formulation F2 of **D55** is equivalent to the 10 mg/mL solution tested in **D33** (cf. **D33**, par. 2 and 4 with **D55**, table 1). Figure 1 of **D55** shows that, starting from about 0.25% aggregate, after 12 weeks (i.e., three months) under accelerated conditions (**D55**, par. 6) about 1.1% to 1.2% of aggregate has formed. It is readily apparent that the behavior observed for formulation F2 in **D55** more closely resembles the aggregate formation of the 30 mg/mL solution of **D33** than the aggregate formation tendency of the 10 mg/mL solution of **D33**.

112. This sheds considerable doubt on the reliability of the result provided by Dr. Miano and further suggests that the differences in the amount of aggregation observed in **D33** between the different concentrations are more likely to result of experimental noise than from actual, significant differences in antibody stability.

113. We further note with regard to Fig. 3 of **D55** that formulation F9, tested at pH 7.5 contained an amount of about 1.5% antibody aggregate after being stored for 12 weeks under accelerated conditions. This is a slightly higher amount of aggregation than what was observed for the 30 mg/mL solution after 12 weeks in **D33** (under accelerated conditions, **D33**, Fig. 1, cf. above), which was about 1.4% aggregate.

114. Dr. Miano in **D33** refers to the 30 mg/mL solution tested therein as not being suitable (**D33**, par. 8) and the Appellant, referring to **D33**, has declared that the 30 mg/mL solution would not meet the release standards after 36 months of storage (par. 5.3.7 of submission dated March 22, 2018). The Appellant has thus suggested that a formulation of eculizumab which exhibits the aggregation characteristics of the 30 mg/mL solution tested in **D33** would be insufficiently stable for being used in the treatment of PNH. Importantly, this would also apply to formulation F9, tested in **D55**.

115. Accordingly, **D55** clearly suggests that (based on the Appellants considerations) an antibody concentration of 10 mg/mL does not solve the problem of either providing a sufficiently stable composition, let alone of providing a "highly stable" composition over the whole scope claimed, i.e., regardless of the solution pH, excipients, etc. To the contrary, formulation F9 is shown in **D55** to be neither sufficiently, nor highly stable, according to the standard suggested by the Appellant.

116. Lastly, all buffers tested in **D55** contain a stabilizing agent known to suppress antibody aggregation. Formulations F1 to F3 and F7 to F9 contain polysorbates, while F4 to F6 contain mannitol (cf. **D55**, table 1).

117. Polysorbates are well known to reduce antibody aggregation, as evidenced by review article **D21**:

> *"Protection from aggregation can be limited in antibody formulations by the addition of surface-active agents such as detergents, both charged and uncharged. As an interesting aside, such surface-active agents can help reduce protein loss due to adherence to surfaces that can occur with low concentration protein formulations. Polysorbate 20 and 80 (now available from a vegetal source) are examples of detergents that are widely used to reduce the rate of protein aggregation in high concentration antibody drug formulations."* (**D21**, p. 693, right col., 2nd par., emphasis added)

118. Mannitol is similarly used to reduce the amount of aggregation in antibody formulations:

> *"Improved native-like structure and a reduction in aggregation can be obtained by incorporation of a carbohydrate excipient in sufficient quantities to fulfill the hydrogen bonding requirements on the protein surface, suggesting a critical role for non-water molecules to act as placeholders for water molecules during drying and in the dry state. That the addition of excess carbohydrate failed to improve the stabilizing function of this excipient, reaffirms the conclusion that antibodies have a limited number of sites that must be protected in this fashion [65]. A variety of carbohydrates or polyol compounds such as sucrose, trehalose and mannitol have been shown to provide this stabilizing function [66,67]."* (**D21**, par. bridging p. 694/695, emphasis added)

119. This means that all tested formulations of **D55** comprise excipients that are known to have a significant influence on antibody aggregation. Nevertheless, the granted claim does not limit the possible excipients in the solution in any way or require any such excipient to be present. The experiments provided in **D55** therefore fail to show that the claimed composition offers sufficient stability against degradation regardless of the excipients (and their amount) being used, let alone a "high" degree of stability. We note at this point that also the solutions tested in **D33** contained a stabilizing agent (cf. **D33**, par. 2, polysorbate 80).

120. The Appellant thus still has not provided convincing evidence that the suggested technical problem has been credibly solved at all by the claimed subject matter, let alone over the whole scope claimed (any amount of any thinkable excipient).

121. As noted above, an Applicant (Appellant in this case) relying on a reformulated technical problem bears the burden of proof that the newly formulated problem has been solved over the whole scope claimed.

122. In summary, the technical problem of providing a highly stable, low-volume composition of eculizumab for the treatment of PNH has not been credibly solved, in particular not over the

whole scope claimed due to the reasons provided hereinabove and in our previous submissions (sections IV.3 and IV.4 of our submission dated November 21, 2018).

III.3.4 Arriving at the claimed subject matter does not require inventive activity

123. Even if the claimed concentration were considered to solve the purported technical problem over the whole scope claimed, the skilled person would arrive at this concentration in an obvious manner.

124. We have explained in detail in our previous submissions that the skilled person starting from **D1** and taking the disclosure of **D22** (or **D45**) into consideration would have arrived at the claimed subject matter without having to rely on inventive activity (cf. section IV.2 of our submission dated November 21, 2018).

125. The Appellant has explained why in its view, the person of skill in the art would have disregarded the teaching of **D22**. The Appellant's arguments are essentially constructed around the allegation that **D22** would not teach solutions of eculizumab (par. 5.6.4 and 5.6.9 of statement of grounds). This is incorrect.

III.3.4.1. The claimed concentration constitutes an arbitrary choice

126. As we have explained in detail in our previous submissions (section VII.5 of our notice of opposition, section IV.1 of our submission dated November 21, 2018), the concentration of 10 mg/mL does not provide a specific technical effect, since a solution of eculizumab having a concentration of 10 mg/mL fails to provide a technical effect when compared to other concentrations, in particular when compared to any other concentration falling within the range of 1 to 30 mg/mL disclosed in **D22**. The feature *"10 mg eculizumab/ml"* thus constitutes an arbitrary choice made by the skilled person in the context of routine experimentation.

127. In any case, determining solubility and stability is part of routine experimentation in protein formulation and does not involve any inventive activity.

128. We refrain from repeating all arguments provided in our previous submissions in detail for the sake of brevity. Briefly, the determination of a suitable antibody concentration is part of the routine experimentation, the skilled person would perform when developing an antibody formulation. As we have noted before, a suitable concentration has to be determined in any case. Accordingly, the skilled person starting from **D1** would under any circumstances have performed assays to identify a concentration that provides sufficient stability of the antibody of choice, in this case eculizumab.

129. Bearing this in mind, the concentration of 10 mg/mL is comfortably within the common range of concentrations for pharmaceutical antibody formulations (**D20**, **D21**, cf. section IV.2 of our submission dated November 21, 2018). The skilled person would further have been aware from **D22** that specifically eculizumab may be formulated at concentrations ranging from 1 mg/mL to 30 mg/mL (**D22**, Fig. 10 and par. [0170] to [0172]) and would then be at least sufficiently stable and biologically active to be used as a drug. The Appellant alleges in its statement of grounds of appeal that the skilled person would not have taken the teaching of **D22** into consideration. This allegation is unfounded, as discussed in detail below.

130. The burden of proof to show that the concentration of 10 mg/mL results in a specific technical effect lies with the Appellant (cf. supra). In particular in the present case, in light of the common general knowledge (higher concentration leads to faster aggregation), the Patentee should be required to show that 10 mg/mL is more stable than other concentrations that are higher or lower.

131. With regard to the effect of antibody concentration on the aggregation propensity of an antibody, **D21** teaches:

"As protein–protein contact frequency increases at high concentrations, the opportunity for stable aggregate formation increases proportionally. […] As protein concentration and temperature goes up, so does the probability of energetically favorable contacts leading to aggregation with increased frequency and duration of reversible protein–protein contacts leading to a greater extent of stable (even irreversible) aggregation events. Aggregation (covalent, non-covalent, dissociable, non-dissociable) must be considered a likely degradation event for high concentration antibody formulations." (**D21**, par. bridging p. 692/693, emphasis added)

132. Based on common general knowledge, the skilled person would expect aggregation to increase proportionally with antibody concentration.

133. We have provided evidence that concentrations lower than 10 mg/mL provide a long-term stability of the antibody that is at least as good as for 10 mg/mL. Based on common general knowledge, this is exactly what the skilled person would expect. The skilled artisan's expectation is confirmed by **D23**.

134. Tellingly, the Appellant has so far not provided any evidence showing that a solution of eculizumab at 10 mg/mL is more stable than lower concentrations. All of the data the Patentee has provided compares 30 mg/mL to 10 mg/mL. While we maintain that the concentration of 10 mg/mL does not offer any advantage over the concentration of 30 mg/mL (cf. **D23**), there is no evidence whatsoever that the concentration of 10 mg/mL would be beneficial when compared to, e.g., 5 mg/mL or 2 mg/mL.

135. This is highly relevant because, as explained above, based on common general knowledge, the skilled person would expect lower concentrations to offer at least equivalent long-term stability (with regard to aggregation) and we have provided evidence that this is the case. Even if the three declarations by Dr. Dino Miano and the data disclosed therein are accepted to provide a valid proof that 10 mg/mL is beneficial over 30 mg/mL (which we do not), no information is derivable from the expert declarations with regard to lower concentrations.

136. We further note that even when compared to higher concentrations, the claimed concentration does not offer any advantage. **D25**, a patent application filed by the Patentee, shows that high concentration formulations (up to more than 100 mg/mL) of eculizumab are equally stable, compared to 10 mg/mL and maintain biologic activity of the antibody over at least 24 months (cf. sections 39 to 46 of our submission dated November 21, 2018). **D25** thus provides evidence that in terms of antibody aggregation, and particularly with regard to biological activity, the concentration of 10 mg/mL offers no advantage.

137. It is important to note that the Miano declarations provide no basis to refute this evidence. As we have explained in detail before, the loss in activity of an antibody formulation occurs via multiple pathways, some of which are interdependent, such as denaturation, aggregation, surface adsorption, chemical degradation (oxidation, deamidation, formation of acidic species), etc. This has been correctly noted by the Appellant under section 4.3.3 of the submission dated March 22, 2018. One particular degradation pathway does not necessarily dominate for any one antibody (see also section 4.3.4 of the submission dated March 22, 2018). Nevertheless, Dr. Miano has solely analyzed aggregation of the antibody at merely two different concentrations (10 mg/mL and 30 mg/mL) in **D33**.

138. Dr. Miano's second declaration, **D54**, allegedly shows that the concentration of 10 mg/mL is beneficial in terms of the biological activity of the antibody formulation. As explained above, it fails to do so. According to **D54**, antibody dimers have been isolated from a sample that had been stored for 52 months (par. 5 of **D54**). Since eculizumab had originally been marketed with a shelf-life of 24 months (see FDA letters annexed to **D33**, **D24** at p. 6, under *"How Supplied/Storage and Handling"*), this means that the analyzed sample would have in any case been discarded more than two years before Dr. Miano has used it for his analysis. This already casts doubt on the relevance of the results in the context of the opposed patent.

139. As we have noted above, the case law of the Boards of Appeal is clear in that the formulation of artificial problems is to be avoided when analyzing inventive step. Analyzing a solution of eculizumab after a storage time of 52 months is completely artificial in relation to the marketed product and even more so in relation to the technical problem formulated in the original disclosure.

140. If a sample had been stored over a duration of, e.g., 200 months and then been analyzed with regard to aggregate formation would this have any relevance in the context of the opposed

patent? The answer must clearly be negative in our view. It should be kept in mind that the original disclosure is aimed at the provision of a treatment for patients suffering from PNH using an antibody that binds C5 (**WO**, p. li. 24 to 28). Any inventive step discussion must be within the limits set by the original disclosure, which does not relate to maximizing long-term stability of eculizumab formulations.

141. Moreover, Dr. Miano has isolated antibody dimers from the 52-month-old solution of eculizumab and then tested the isolated aggregates for their biological activity. Since antibody solutions degrade via multiple pathways and it is not possible to predict which of said pathways will be predominant, it is entirely unclear whether the 10 mg/mL solution indeed had a greater overall biological activity than a solution having a concentration of 30 mg/mL. This question simply cannot be answered based on the data provided by the Appellant. The purification method adopted by Dr. Miano (size exclusion chromatography) solely differentiates analytes based on their size (more precisely, hydrodynamic volume). The method cannot be used to separate molecules of the same size that differ only with regard to their chemical structure (e.g., acidic species of the same antibody) or activity. However, chemical degradation may have a significant impact on the biological activity of an antibody solution.

142. The fact that Dr. Miano has determined the purity of the tested solutions solely by SEC means that no reliable conclusion can be made based **D54** with regard to the overall loss in biological activity of the tested samples. It is to be expected that chemical degradation of eculizumab takes place in parallel to aggregation but it is unclear to which degree this is influenced by the concentration of the antibody. It therefore cannot be followed from the data provided by the Patentee in **D54** that a solution of eculizumab having a concentration of 10 mg/mL exhibits a higher biological activity after prolonged storage than a solution having a concentration of 30 mg/mL. This has simply not been shown.

143. As noted above, **D54** offers no direct comparison of two solutions of eculizumab at 30 mg/mL and 10 mg/mL. **D25** in contrast directly compares solutions of eculizumab having different concentrations at different time points and shows that there is no difference. Due to the skewed experimental setup, **D54** cannot disprove the teaching of **D25**. In addition, **D23** includes a rCE-SDS analysis, which, as we have noted previously, offers a reliable overall picture with regard to the formation of physically and chemically degraded species (aggregates, fragments, acidic species, etc.) (**D23**, Appendix C; par. 54 of our notice of opposition). This analysis shows that there is no significant difference between the tested concentrations of 50, 30, 10, 5 or 2 mg/mL.

144. In summary, we have provided evidence (**D23** and **D25**) that the concentration of 10 mg/mL offers no benefit over other concentrations (higher or lower) with regard to aggregation and chemical degradation (**D23** and **D25**) or biological activity (**D25**). The Patentee has failed to provide evidence that would disprove the teaching of **D23** and **D25**. In particular, there is no

evidence on file showing that a 10 mg/mL solution of eculizumab provides a higher biological activity when compared to a solution with a concentration of 30 mg/mL. In the context of the original disclosure, a technical effect that is confined to an allegedly slower aggregation cannot be taken into consideration for reformulating the technical problem, as explained above.

145. The Appellant states in this context (cf. par. 5.2.5 of statement of grounds of appeal) that the purportedly higher monomer content of the 10 mg/mL eculizumab formulation would be *"of significant benefit to the patient"*. This is a mere allegation that is completely devoid of any experimental basis. No data has been provided which would show that the claimed formulation actually offers a benefit of any kind to the patient suffering from PNH.

146. Moreover, the Patentee has not even tried to show that the concentration of 10 mg/mL is beneficial over lower concentrations. This is a significant omission, since **D23** clearly shows that lower concentrations are at least as stable as 10 mg/mL and this is in line with common general knowledge. Based on the evidence on file, it therefore cannot be concluded that 10 mg/mL actually offers any advantage, i.e., provides any specific technical effect. The concentration of 10 mg/mL thus constitutes an arbitrary choice.

III.3.4.2. The skilled person would arrive at the claimed concentration in an obvious manner

147. We have explained in detail in our previous submissions that the skilled person starting from **D1** and taking the disclosure of **D22** (or **D45**) into consideration would have arrived at the claimed subject matter without having to rely on inventive activity (cf. section IV.2 of our submission dated November 21, 2018).

148. The Appellant has explained why in its view, the person of skill in the art would have disregarded the teaching of **D22**. Again, the Appellant's arguments are essentially constructed around the allegation that **D22** would not teach solutions of eculizumab (par. 5.6.4 and 5.6.9 of statement of grounds). This is incorrect.

149. The OD has correctly noted that the relevant question in the current case, i.e., in light of the original disclosure, is whether **D22** provides the skilled person with information regarding formulations of eculizumab that are sufficiently stable and suitable for being administered to a patient. This is the case.

150. **D22** teaches that eculizumab may be formulated at concentrations between 1 mg/mL and 30 mg/mL (**D22**, Fig. 10 and par. [0170] to [0172]). Importantly, even the buffers disclosed as being suitable in **D22** encompass the buffer used in the commercial formulation of eculizumab (Fig. 10, **D22**: 10 mM sodium phosphate, 150 mM sodium chloride, pH 6-8 and 0.001 to 0.02% Tween 80, compare section 2 of **D33**). "Tween 80" is synonymous for polysorbate 80 (see also [0069] of **D22**).

151. **D22** also discloses that eculizumab maintains its integrity after being formulated according to the teaching of **D22** and subsequent nebulization, as shown by SDS-PAGE and HPLC ([0173] of **D22**). **D22** thus teaches that eculizumab, when being formulated as described therein is sufficiently stable to be used as a drug.

152. Moreover, subsequent to nebulization, the eculizumab formulation disclosed in **D22** is analyzed by HPLC (**D22**, [0173]). As the skilled person is well aware, only solutions that are free from particulate matter can be analyzed by HPLC, because otherwise, the densely packed stationary phase of the HPLC columns will clog and eventually be damaged. Solid particles will in any way not pass through an HPLC column and therefore cannot be analyzed by HPLC. It is thus clear to the skilled person that the formulation disclosed in **D22** can impossibly be a suspension or a slurry (cf. statement of grounds of appeal, par. 5.6.5). Instead it must have been a solution. This is immediately apparent to the skilled person.

153. The Appellant's analysis with regard to the question the skilled person would be faced with is furthermore incorrect. As rightly determined by the OD, the question the skilled artisan is faced with, would be finding a suitable concentration. Other features of the granted claim, such as the medical indication (PNH), or the dose are taught or implied by **D1**. Contrary to the Appellant's allegations, the skilled person would not be faced with the problem of providing a low volume liquid composition having good antibody stability, but with the task of providing a formulation of eculizumab which is suitable for being used as a drug for the treatment of PNH. Faced with this task, the skilled person would take from **D22** that eculizumab, formulated as disclosed in **D22** would maintain its integrity in solution, i.e., would be sufficiently stable to be used as a drug.

154. The Appellant tries to generate the impression that the skilled person would disregard the teaching of **D22**, merely due to the fact that it is directed at formulations intended for nebulization. This is surprising in light of the original disclosure, which states:

> *"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."* (**WO**, p. 25, li. 6 to 9, emphasis added)

155. Thus, the original disclosure, which is directed to providing treatments for PNH, by no means excludes administration by inhalation. It is unclear what would have kept the person of skill in the art from taking this teaching into consideration. **D22** teaches solutions of eculizumab that are sufficiently stable to be used as a drug. This is exactly the information the skilled person would be looking for.

156. The Appellant's discussion of nebulizer efficiency, respirable fraction (par. 5.6.11) or salbutamol formulations (par. 5.6.15) is completely immaterial in this context, because the

skilled person would be faced with the specific question of identifying a suitable concentration for eculizumab, as discussed above. **D60**, a post-published drug guideline issued by the Australian Liverpool Hospital regarding the drug salbutamol and its use in this hospital, is irrelevant. It is not part of the common general knowledge (let alone at the relevant date), not referred to in the original teaching or the closest prior art. It further relates to a completely different active agent. It is unclear in which way **D60** relates to the relevant questions to be discussed in these proceedings.

157. Starting from **D1**, in combination with either **D22** (or **D45**, cf. our submission dated November 21, 2018) the skilled person would have had a reasonable expectation of success that a pharmaceutical composition comprising eculizumab in a concentration of 10 mg/mL would be suitable for the treatment of PNH.

158. Even if this were rejected by the Board, we submit that the skilled person would have adopted a try-and-see attitude and would have tested eculizumab formulations using the buffers disclosed in **D22** within a concentration range between 1 and 30 mg/mL. With a limited amount of routine experimentation, the skilled artisan would have arrived at a concentration of 10 mg/mL, even if it is assumed that a particular technical effect is provided by this concentration (which is not the case). We refer to section IV.2 of our submission dated November 21, 2018.

159. Thus, the claimed subject matter is obvious in view of **D1** in combination with **D22**, or **D45**, even if it is assumed that the claimed concentration results in a technical effect (which it does not), due to the reasons laid out herein and in our previous submissions (section VII.5 of our notice of opposition and section IV.2 of our submission dated November 21, 2018).

III.3.5 *The antibody sequence disclosed in the opposed patent cannot form the basis of an inventive step*

160. We have explained *supra* why the allegedly unknown sequence of eculizumab cannot be taken into consideration for the reformulation of the technical problem starting from **D1**. Should the Board disagree with this assessment and consider that the sequences disclosed in SEQ ID NOs 2 and 4 may be used as distinguishing features for the granted claims in view of **D1** as the closest prior art, we submit that no inventive step arises from this allegedly distinguishing feature.

161. The granted claim refers to a pharmaceutical formulation comprising eculizumab. At the same time, the Appellant alleges that the skilled person would understand that eculizumab is an antibody consisting of SEQ ID NOs 2 and 4. Thus, according to the Appellant, the granted claims relates to a pharmaceutical composition comprising an antibody that consists of a heavy chain having the amino acid sequence of SEQ ID NO 2 and a light chain having the

amino acid sequence of SEQ ID NO 4. This is the antibody that must be present in the claimed formulation, if the Appellant's logic is to be followed.

162. However, the Appellant has not stated which technical effect would arise from the amino acid sequence of SEQ ID NOs 2 and 4. We note that neither the clinical studies on file, nor the experiments by Dr. Miano have been executed using an antibody comprising the light chain amino acid sequence of SEQ ID NO 4.

163. **D33**, the first declaration of Dr. Miano teaches that the tests were performed using a 30 mg/mL formulation of eculizumab which is identical to the commercial formulation of eculizumab, except for the concentration (**D33**, par. 4). Of course, the commercial formulation of eculizumab does not contain an antibody having a light chain with an amino acid sequence of SEQ ID NO 4. The experiments of **D33** were thus not performed with the antibody that (according to the Appellant) is required by the granted claim.

164. This also applies to **D54** (par. 3). Since **D55** refers to **D33** and **D54**, it must be assumed that the experiments disclosed in **D55** have also been performed using the commercial antibody.

165. Accordingly, there is no evidence on file that would relate to the properties of an antibody consisting of SEQ ID NOs 2 and 4 and the Appellant has not explained which technical effect would arise from this specific antibody.

166. It has thus not plausibly been demonstrated that an antibody defined by SEQ ID NOs 2 and 4 is useful for the treatment of PNH, or that it would possess any other property which could be used as a basis for acknowledging an inventive step.

167. Based on the Appellant's submissions, it is therefore not credible/plausible that an antibody defined by SEQ ID NOs 2 and 4 solves the technical problem put forward by the Patentee, or, in fact any other technical problem. As noted above and in our previous submissions, the Appellant bears the burden of proof in this regard.

168. It should further be taken into consideration that the EPO routinely rejects claims to antibodies that are solely rendered novel based on their amino acid sequence, for lack of inventive step. In the absence of any surprising technical effect or functional property, the sole provision of an alternative antibody having a different amino acid sequence, but similar properties to the antibodies known from the prior art is not considered to be inventive. The skilled person is considered to have access to antibodies with desired properties by means of routine experimentation. As noted above, the Appellant has failed to provide such a surprising technical effect for an antibody defined by SEQ ID NOs: 2 and 4 of the opposed patent.

169. Thus, the sequences of SEQ ID NOs: 2 and 4 cannot constitute a feature that gives rise to an inventive step.

III.4 Lack of sufficient disclosure (Article 100(b) EPC)

170. The OD considered the granted claims to be compliant with Article 83 EPC, because the skilled person would be able to produce a formulation according to the granted claim (cf. section 2.3.2 of the decision).

171. We disagree with this assessment. The OD has stated that (in agreement with the position of the Appellant), the granted claim is directed to the therapeutic use of the claimed composition. In such a case, the disclosure of the patent is not rendered sufficient by merely disclosing the manufacture of the claimed composition in a manner compliant with Article 83 EPC.

172. For medical use claims, attaining the claimed therapeutic effect is a functional technical feature of the claim (CLBA, II.C.6.2). As a consequence, unless this is already known to the skilled person at the priority date, the application must disclose the suitability of the product to be used for the claimed therapeutic application.

173. Accordingly, the opposed patent must provide sufficient evidence to render it plausible that an antibody covered by the granted claim may be used for the treatment of PNH, i.e., the opposed patent must provide sufficient evidence that the claimed therapeutic effect can be achieved using the claimed active agent(s).

174. Given that the TRIUMPH study disclosed in the opposed patent (**B1**, [0070], cf. also **D18**) was a phase III clinical study approved by the regulatory agencies it may be assumed that it was performed using the antibody sold under the trade name Soliris®. This would mean, however, that the clinical data disclosed in the opposed patent does not actually relate to the antibody defined by SEQ ID NOs: 2 and 4 of the opposed patent, because the (correct) light chain antibody sequence of Soliris® is not identical to SEQ ID NO: 4 (cf. par. 60 and 61 of our notice of opposition).

175. The opposed patent therefore fails to render it plausible that the claimed therapeutic effect can be achieved using the antibody encompassed by the granted claim, if the Board is minded to follow the Appellant's argument and accept that the term "eculizumab" as used in the granted claim specifies the encompassed antibody to consist of amino acid sequences defined by SEQ ID NOs: 2 and 4. The claimed subject matter is thus not sufficiently disclosed.

IV. The Auxiliary Requests

IV.1 Admissibility

176. We generally note that the Auxiliary Requests filed by the Appellant fail to be convergent. The ARs are either aimed at restoring inventiveness by defining the antibody sequence in two

mutually contradictive ways (ARs 1, 2, 4 and 5), by defining the pH of the composition (ARs 3 to 5 and 7), by defining the mode of administration (ARs 6 to 8) or by specifying a dilution step prior to administration (AR8).

177. The ARs thus limit the claimed subject matter in different, mutually unrelated ways, some of them even relating to different technical problems.

178. Already for this reason, the set of ARs submitted by the Appellant should not be admitted to the proceedings.

IV.1.1 *Auxiliary Requests 1, 3 and 4*

179. AR1 submitted by the Appellant with the statement of grounds of appeal corresponds to AR1B as filed during oral proceedings at the opposition stage.

180. The OD decided to not admit AR1B (now AR1) into the proceedings because it was firstly not convergent with the further request submitted by the Patentee during oral proceedings (AR1A, now AR3) and secondly, because arguments taking this feature into account had only been brought up by the Patentee on the day of the oral proceedings (cf. section 5.2 of decision revoking the European Patent). According to the OD, the analysis of the feature introduced at such late stage proved to be complex (*ibid.*).

181. The very same request was now resubmitted by the Appellant at the appeal stage. We note that when a decision needs to be taken by a Board of Appeal on a request that had already been refused by the OD, this amounts to a review of the OD's exercise of its discretion under Rule 116(2) EPC, as opposed to a review of all the facts and circumstances of the case as if it were in that department's place and to decide whether or not it would have exercised such discretion in the same way.

182. The exercise of discretion by the first instance should only be overturned by the board if wrong criteria were applied, or if the discretion was exercised unreasonably:

> *"If a first instance department is required under the EPC to exercise its discretion in certain circumstances, such a department should have a certain degree of freedom when exercising that discretion, without interference from the Boards of Appeal. In the circumstances of a case such as that before the referring Board, a Board of Appeal should only overrule the way in which a first instance department has exercised its discretion if it comes to the conclusion either that the first instance department in its decision has not exercised its discretion in accordance with the right principles as set out in paragraph 2.5 above, or that it has exercised its discretion in an unreasonable way, and has thus exceeded the proper limits of its discretion."* (**G 7/93**, reasons 2.6)

183. While in **G 7/93** a decision of the Examining Division was under appeal, the principles laid out therein have been widely applied by the Boards of Appeal to opposition appeal proceedings

(cf. **T 28/10**, reasons 2.1, but also more recent decisions such as **T 241/13**, reasons 2.1 and **T 2000/15**, reasons 4.1 to 4.4).

184. An admission of AR1 and AR3 into the proceedings would thus only be justified if the board came to the conclusion that the OD had improperly exercised its discretion. We note in this regard that the Appellant has failed to present any arguments why this should be the case.

185. The criteria that should be taken into account by an OD when deciding on the admissibility of a late-filed request include *prima facie* allowability, procedural expediency/abuse of procedure and reasonable expectation that the opponents familiarize themselves with the proposed amendments in the time available (**T 491/09**, reasons 1.4 to 1.6, cf. also Guidelines for Examination E-III 8.6).

186. In agreement with these requirements, the OD has analyzed the *prima facie* allowability of the amendments introduced in AR1A and AR1B during oral opposition proceedings and came to the conclusion that the Auxiliary Requests lacked *prima facie* allowability (cf. sections 4.2 and 5.2 of decision revoking the European Patent). The OD further noted that the feature introduced into AR1B (now AR1) warranted complex analysis and thus agreed with the Opponents in that they could not be reasonably expected to analyze the newly formed feature combination to the degree necessary to properly address the late filed requests.

187. We have explained above that the additional reference to SEQ ID NOs: 2 and 4 does not contribute in any way to solving the technical problem the Appellant has put forward during written opposition proceedings, but instead opens an entirely new discussion relating to a new (partial) problem – the provision of an alternative antibody. This represents another reason why AR was correctly rejected by the OD, since it goes against procedural economy and is not *prima facie* allowable.

188. Procedural abuse may lie, e.g., in the submission of multiple requests that fail to be convergent, which essentially confronts the OD with the task of determining the patentable content of the patent in suit. The OD has correctly considered that AR1A and AR1B as filed during oral proceedings failed to be convergent and thus led to a situation as described above. This constituted another sound reason to refuse the late-filed requests.

189. In this context, we refer again to the Appellant's submission dated March 22, 2018 (section 5.2.1) and the submission dated November 21, 2018 (section 3.4), wherein the differences over the prior art were defined with no mention of the allegedly unknown antibody sequence. As explained above, **D57** represents evidence that the Appellant was considering that the sequence of eculizumab was not disclosed to the public at the filing date of the opposed patent, in particular, that neither **D3** or **D13**, nor **D17** disclosed the sequence of the antibody (**D57**, par. 3 and 4). As we and Opponent 2 have explained previously and hereinabove, the disclosure of **D3** is highly similar in its essential parts to the disclosure of **D1**. Thus, while the Appellant has in 2016 argued in US patent proceedings that the sequence of eculizumab was

not known at the filing date, Alexion decided not to use this argument in their written submissions during opposition proceedings in the present case.

190. However, on the day of oral proceedings, the Patentee surprisingly stated that the patent in suit would, for the first time, disclose the full-length amino acid sequence of eculizumab and this would constitute a further difference over **D1**. Given that **D1** was used as the starting point for the inventive step analysis by both the opponents and the OD and that the very same document had already been cited as closest prior art by the Examiner during prosecution of the patent (cf. ESOP of July 21, 2011), this must lead to the conclusion that the Proprietor purposefully decided to withhold the argument until the very last moment to take the Opponents (and the OD) by surprise.

191. Such conduct is a clear abuse of procedure, which represents yet another reason why the OD rightfully decided not to admit AR1B (now AR1) into the proceedings.

192. During Appeal proceedings, the parties have only limited scope to amend the subject of the dispute in relation to first-instance proceedings, and this principle is reflected in Art. 12(4) RPBA. The appeal proceedings are not about bringing an entirely fresh case. However, this is exactly what the Appellant tries to do by filing ARs 1, 2, 4 and 5.

193. The intention behind this strategy may be assumed to be a shift of the discussion with regard to Article 56 EPC from the concentration of 10 mg/mL to the allegedly formerly undisclosed sequence of eculizumab. This would amount to bringing an entirely fresh case, since prior to oral opposition proceedings the proprietor has, presumably based on strategic considerations, never relied on the sequence of the antibody as a distinguishing feature (cf. above).

194. In summary, ARs 1 and 3 should thus not be admitted into the proceedings under Article 12(4) RPBA.

195. AR 4 represents a combination of the features introduced into ARs 1 and 3 and should therefore be refused for the same reason.

IV.1.2 Auxiliary Requests 2 and 5

196. ARs 2 and 5 also rely on the feature of SEQ ID NOs: 2 and 4 as a distinguishing feature, in a modified form. The Appellant has introduced SEQ ID NO: 2 in full and SEQ ID NO: 4 as a shortened fragment that lacks the 22 amino acid signal peptide. This fragment is nowhere disclosed in the original teaching, as will explained in more detail below. In any case, the introduction of the SEQ ID NOs: 2 and 4 either fully or in part, constitutes a procedural abuse due to the reasons provided above.

197. In addition, it is clear that the parent application as filed can impossibly provide a clear and unambiguous basis for both, an antibody termed “eculizumab” defined by SEQ ID NOs: 2 and 4 and an antibody termed “eculizumab” defined by SEQ ID NO: 2 and residues 23 to 214 of

SEQ ID NO: 4. This will be discussed in further detail below. ARs 1, 2, 4 and 5 are therefore obviously not convergent in their definition of the claimed antibody, which represents yet another violation of Rule 80 EPC.

198. ARs 2 and 5 should therefore not be admitted into the proceedings by the Board.

IV.2 The Auxiliary Requests introduce new violations of the EPC and fail to solve the problems that arose during opposition proceedings

IV.2.1 Auxiliary Request 1

IV.2.1.1. Added Matter (Art. 100 (c) EPC / Art. 76 (1) EPC, 123(2) EPC)

199. AR1 suffers from the same deficiency under Article 123(2) EPC as the Main Request (cf. above).

200. The SEQ IDs introduced in to the claim of AR1 are allegedly derivable from **WO**, p. 5, ultimate par. and SEQ ID NOs 2 and 4 of the original disclosure. The referred to passage corresponds to par. [0018] of the granted patent, discussed above. Due to the same reasons provided *supra* with regard to the disclosure of the granted patent, the original filing does not teach in a direct and unambiguous manner that "eculizumab" is an antibody defined by SEQ ID NOs: 2 and 4. For this reason, AR1 violates Article 123(2) and 76(1) EPC.

201. The Appellant states in par. 6.2.1 of the statement of grounds that *"the sequence of eculizumab"* has been inserted into the claim of AR1. In contrast to this allegation, the Appellant states in par. 6.3.2 that the skilled person would immediately recognize that SEQ ID NO: 4 contains an error and that it was the Applicant's intention to define the antibody as including only residues 23 to 214 of SEQ ID NO: 4. These statements are mutually contradictive. Either the original disclosure teaches that eculizumab is an antibody defined by SEQ ID NOs: 2 and 4 or it teaches that eculizumab is defined by SEQ ID NO: 2 and residues 23 to 214 of SEQ ID NO: 4 (or it fails to disclose any of the two).

202. The parent application as filed can impossibly provide basis for both variants. Thus, either ARs 1 and 4 or ARs 2 and 5 must violate Articles 123(2) and 76(1) EPC.

IV.2.1.2. Inventive Step (Article 100(a) EPC / Article 56 EPC)

203. The arguments provided above with regard to the Main Request also apply to AR1.

204. There is no evidence on file that would relate to the properties of an antibody consisting of SEQ ID NOs 2 and 4 and the Appellant has not explained which technical effect would arise from this specific antibody.

205. It has not plausibly been demonstrated that an antibody defined by SEQ ID NOs 2 and 4 is useful for the treatment of PNH, or that it would possess any other property which could be used as a basis for acknowledging an inventive step. The Appellant has not provided any arguments in this regard.

206. Based on the Appellant's submissions, an antibody defined by SEQ ID NOs 2 and 4 therefore fails to solve a technical problem and thus cannot constitute a feature that gives rise to an inventive step.

207. AR1 thus does not alleviate any of the deficiencies of the MR under Article 56 EPC.

208. Similar to the MR, AR1 hence lacks an inventive step in view of **D1** alone or in combination with **D22** or **D45**.

IV.2.1.3. Lack of sufficient disclosure (Article 100(b) EPC)

209. We have explained above why the granted claim, i.e., the claim of the Main Request, lacks sufficient disclosure. AR1 lacks sufficient disclosure due to the same reasons.

210. The antibody defined by SEQ ID NOs: 2 and 4 of the opposed patent is not Soliris®, i.e., it is not identical to the antibody that was used in the clinical study disclosed in the opposed patent, or in any other of the numerous clinical studies disclosed in the cited documents in this case.

211. There is no evidence on file which would render it plausible that the claimed therapeutic effect of treating PNH can be achieved using the specific antibody recited in AR1.

212. Therefore, AR1 is insufficiently disclosed.

IV.2.2 Auxiliary Request 2

IV.2.2.1. Added Matter (Art. 100 (c) EPC / Art. 76 (1) EPC, 123(2) EPC)

213. It is not disputed that an antibody comprising a light chain amino acid sequence as defined by residues 23 to 214 of SEQ ID NO: 4 is not explicitly disclosed in the original teaching. The Appellant nevertheless suggests that it is derivable from the sequence taught in SEQ ID NOs: 3 and 4 of the international application in a direct and unambiguous manner that it was the applicant's intention to define the antibody as including the mature light chain sequence, which is defined by residues 23 to 214 of SEQ ID NO: 4.

214. According to the Appellant, the skilled person would have immediately recognized that the light chain sequences shown in SEQ ID NOs: 3 and 4 comprise a 22 amino acid signal peptide at the N-terminus that would be cleaved off during antibody production. We disagree.

215. As noted above, the original teaching can only provide direct and unambiguous disclosure for one of the two alternatives, provided by the Appellant in the form of AR1 and AR2. At least one of the two variants must lack original disclosure.

216. The amendment introduced into claim 1 of AR2 would only be compliant with Articles 123(2) and 76(1) EPC, if the skilled person would recognize that there is an error in the sequence disclosed in the specification and if he/she could directly and unambiguously recognize the exact sequence signal peptide that was erroneously incorporated. If the skilled person was in doubt with regard to the correct starting point of the mature antibody sequence, the restriction to residues 23 to 214 of SEQ ID NO: 4 would confront the skilled person with technical information that is not originally disclosed.

217. In support of its allegations, the Appellant has filed **D59**, a declaration of Dr. Bruce Andrien. The expert explains why it was allegedly obvious to the skilled artisan that SEQ ID NO: 4 contains a signal peptide that is exactly 22 amino acids in length.

218. Notably, Dr. Andrien discusses the teaching of **D38** with regard to only the sequences of signal peptides having a length of 22 amino acid (**D59**, section 11). This approach is clearly based on hindsight. How would the skilled person know (beyond doubt) that the signal peptide that was erroneously included in SEQ ID NOs: 3 and 4 has a length of exactly 22 amino acids? Even if the skilled person would recognize that SEQ ID NOs: 3 and 4 comprises a signal peptide, the skilled person would subsequently be confronted with the question of how long the signal peptide was. The skilled person would thus have to consider signal peptides of any length instead of starting his/her analysis on the assumption that the signal peptide had a length of 22 amino acids.

219. According to the expert, signal peptides of 22 amino acids length "predominantly" start with Met-Asp-Met and "predominantly" end with Arg-Cys. With regard to the first residues of the mature sequence, Dr. Andrien states that he started his analysis with subgroup I of human kappa light chains *"because it was known that subgroup 1 outnumbers each of the other subgroups in usage"* (**D59**, section 12). Apparently, based on this consideration, the expert disregarded the other subgroups in his analysis.

220. Within subgroup 1, the expert notes that the *"vast majority of human kappa light chains catalogued in 1991 started with this sequence"* (emphasis added). It is clear from this statement that even within this isolated subgroup, only a fraction (the "majority") of the mature sequences start with the correct sequence and that this analysis is based on the sequences catalogued in 1991. Lastly, the expert discusses statistics to show that the first four residues of

the mature amino acid sequence comprised in SEQ ID NOs: 3 and 4 of the opposed patent were the most likely candidates.

221. We firstly note that the language used by the expert makes clear that the relevant standard is not met in this case. The relevant question is whether the skilled artisan could derive in a direct and unambiguous manner from SEQ ID NOs: 3 and 4 that these sequences include a signal sequence which must have a length of 22 amino acids and consequently, the mature sequence starts with the four amino acids DIQM. Only then would the skilled person disregard the explicit teaching of the original disclosure. This cannot be a question of likelihood.

222. According to **D59**, the person of skill in the art would firstly consider it likely that he/she is confronted with a human kappa light chain of subgroup I, because this allegedly is the most common subgroup. Secondly, the skilled person would consider it likely that the signal peptide would end with Arg-Cys and that the mature sequence would start with DIQM.

223. We do not believe that the opinions expressed by Dr. Andrien support the view that the amendment submitted by the Applicant in AR2 is derivable in a direct and unambiguous manner from the original teaching.

224. We lastly note that the *Kabat et al.* Reference (**D38**), as submitted by the Appellant, represents only a small selection from an amino acid sequence compendium that itself represents only a selection from the vast space of protein, or antibody sequences. It cannot provide evidence that nothing else than the sequences disclosed therein would have been thinkable sequences of signal peptides, or mature human kappa light chains for the skilled person.

225. This is aggravated by the fact that the 5th edition of Kabat *et al.* was published in 1991, i.e., 16 years prior to the filing date of the international application giving rise to the opposed patent. In the quickly moving field of pharmacologically active antibodies, the space of known antibody sequences has significantly expanded within this period and thus, the sequence compendium referred to by the Applicant was long outdated at the priority date. The number of mature light chain sequences known in the art was certainly much larger in 2007, compared to 1991, accordingly, the number of possible options for the length and/or sequences of signal peptides was likely much larger in 2007, compared to 1991. Thus, the skilled person would have been confronted with even more uncertainty regarding the sequence and length of the signal peptide comprised in SEQ ID NOs: 3 and 4, even if recognizing that the sequences disclosed therein are immature.

226. Thus, Kabat *et al.* is not representative of the entire space of possible sequences the skilled person would have had to consider at the relevant date.

227. It is notable that the Appellant has attempted to introduce the limitation to residues 23 to 214 of SEQ ID NO: 4 as a correction under Rule 139 EPC in two pending divisional cases of the opposed patent (EP 3 167 888 and EP 3 124 029). Anonymous TPOs have been filed in these cases raising doubts regarding the allowability of such a correction.

228. In the observations, the third party has referred to an expert declaration filed in a case the Applicant (and Appellant in this case) has referred to, wherein the expert has discussed typical signal peptide structures (section 12 of the TPOs, see **D62**). As the third party explains, based on this declaration by Tom Vink, the amino acid sequence of SEQ ID NO: 4 (and SEQ ID NO: 3) comprises at least one further potential cleavage site marking the possible terminus of a signal peptide and the possible starting point of the mature light chain sequence (cf. **D61**, sections 13 and 14). Accordingly, it was not directly and unambiguously derivable from the amino acid sequence of SEQ ID NOs: 3 and 4 that residues 1 to 22 (must) represent a signal peptide.

229. Under these circumstances, a replacement of the explicit disclosure of the original application (which includes the leader peptide) by a teaching the skilled person can allegedly derive in an implicit manner, based on common general knowledge should not be accepted by the Board.

230. The OD considered that in the absence of evidence to the contrary, it should be assumed that the skilled person would recognize SEQ ID NO: 4 as being immature and that the mature light chain would start with residue 23 of the sequence (p. 6 , 1st par. of the decision). We disagree. It is the Appellant who tries to replace the explicit teaching of the parent application as filed arguing that the skilled person would recognize it as being incorrect and would immediately be able to derive the correct mature sequence from SEQ ID NO: 4. In such as case, the Appellant should be required to provide sufficient evidence that this would indeed be the case.

231. The Appellant has not met this obligation. As explained above, the Kabat reference (**D38**) is outdated and incomplete. The Andrien declaration (**D59**) essentially concludes that the skilled person would have assumed that SEQ ID NOs: 3 and 4 most likely contain a leader peptide and that the mature sequence probably starts with residue 23. This is not sufficient to show that the skilled person would have derived the mature sequence in a direct and unambiguous manner from the original teaching.

232. Therefore, AR2 violates Articles 123(2) and 76(1) EPC.

IV.2.2.2. Inventive Step (Article 100(a) EPC / Article 56 EPC)

233. Even it is accepted that the original teaching discloses the sequence fragment of SEQ ID NO 4 that has been introduced into AR2, the sequences can only give rise to a new partial problem of providing an alternative antibody for the treatment of PNH.

234. As we have noted above, the technical problem as defined by the Appellant is actually a combination of partial problems. The Appellant has not explained how the allegedly unknown sequence of eculizumab would contribute to the problem of providing a highly stable, low volume formulation.

235. The Appellant has not provided any arguments with regard to the properties of the antibody that is defined by SEQ ID NO: 2 and residues 23 to 214 of SEQ ID NO:4 in comparison to the antibody disclosed in **D13** and **D17** (which are referred to in **D1**).

236. If the claim of AR2 is regarded as encompassing one specific antibody that is defined by defined by SEQ ID NO: 2 and residues 23 to 214 of SEQ ID NO:4, the problem arising from this difference over **D1** could be formulated as the provision of an alternative antibody for the treatment of PNH.

237. According to established case law, the could-would approach does not apply when the problem is merely to find an alternative. For example, in decision **T 631/06**, Board 3.3.01 stated:

> *"In a case where the problem to be solved consists merely to provide an alternative, all the information contained in a document is treated equally by the person skilled in the art, notwithstanding whether it is preferred or not, or whether the implementation of some of the said information presents some difficulties. The so called "could-would" approach, applies when the technical problem to be solved relates in the provision of an improvement or in the suppression of disadvantages, not in the provision of an alternative (see Case Law of the Boards of Appeal 5th 2006 I.D.5, page 132, second paragraph)."* (item 2.3.10, emphasis added)

238. In decision **T 674/08**, Board 3.3.02 stated:

> *"The appellant repeatedly argued that the skilled person might have attempted a combination of surfactants, but would not have contemplated the combination according to the invention. This argumentation could not be taken into consideration, because the would-could approach is in general not meaningful when the problem is to find an alternative or further solution. If the problem is purely to find an alternative or further formulation, the skilled person would modify the existing product in any way he could by arbitrary choice".* (item 2.7 of the reasons, emphasis added)

239. We also refer to the Case Law Book, I.D.9.19.8, in this regard, which reports that:

> *"In **T 892/08** the board referred to the established case law whereby, when the technical problem is simply that of providing a further composition of matter or a further method, i.e. an alternative to the prior art, any feature or combination of features already conventional for that sort of composition of matter or method represented an equally suggested or obvious solution to the posed problem. The boards have repeatedly established that the simple act of arbitrarily selecting one among equally obvious alternative variations is devoid of any inventive character (see also **T 311/95**)."* (emphasis added)

240. Hence, when the problem is merely to find an alternative, the skilled person would have modified the prior art in any way they could by arbitrary choice to provide an alternative.

241. Further, in the case of partial problems, such in the present case, has to be established is whether each set of features is separately obvious in the light of the prior art. Thus, it needs to be established whether the provision of an antibody comprising the amino acid sequence of SEQ ID NO 2 and residues 23 to 214 of SEQ ID NO:4 would have been obvious in view of an independent combination of documents, taking into consideration that the could-would approach does not apply in this case.

242. The Appellant alleges in par. 5.5.14 of the statement of grounds that the supposedly unique engineered heavy chain constant region of the commercial antibody eculizumab was not on the market or known to be in clinical studies. This is, of course immaterial in the context of inventive step. The relevant question is whether the prior art would have directed the skilled person to the provision of an antibody consisting of the amino acid sequences SEQ ID NO 2 and residues 23 to 214 of SEQ ID NO:4 of the opposed patent.

243. First of all, the heavy chain constant region of eculizumab was neither unique, nor unknown at the filing date of the opposed patent. Contrary to the Proprietors allegation, there are other antibodies disclosed in the prior art which contain the same engineered heavy chain constant region (see **D15**, e.g., p. 8, point 9); **D16**, throughout the document, e.g., p. 5, 10, 11, etc.; but also **D43**, SEQ ID 67 and **D44** p. 18) (cf. our submission dated par. 71 to 74).

244. Notably, **D42** explicitly teaches that "eculizumab" possesses a chimeric IgG2/IgG4 constant region (**D42**, p. 1279, left col. penultimate par.).

245. Thus, neither the chimeric heavy chain constant region used in SEQ ID NO: 4 of the opposed patent, nor the fact that the marketed antibody eculizumab comprises a chimeric IgG2/IgG4 constant region were unknown at the relevant date.

246. As discussed extensively during opposition proceedings and herein, **D1** refers to **D17**, which teaches the variable regions of the antibody that is marketed as eculizumab.

247. **D1** discloses at par [0012] that eculizumab is also termed h5G1.1. **D17** teaches the variable region sequences of eculizumab as part of an scFv construct termed *"h5G1.1 CO12"* (**D17**, col. 44, 2nd par. and SEQ ID NO: 20). It is further clear from **D17** that the variable region sequences disclosed therein for h5G1.1 (eculizumab) may be combined with suitable IgG constant regions, which may be unaltered, or constructed of a mixture of IgG constant regions (**D17**, col. 45, 3rd par.).

248. Document **D15** relates to monoclonal antibodies that do not activate complement, nor bind to the Fc receptor, in particular the FcRI receptor (**D15**, p. 7, 3rd par.), which are properties that are obviously desirable in the context of the provision of anti-C5 antibodies.

249. To achieve this goal, **D15** discloses *inter alia* recombinant antibody molecules that comprise fragments of the human IgG2 and IgG4 constant regions (termed "HuG2/G4 mAb", **D15**, p. 8, under 9). The recombinant antibodies taught by **D15** include *"a humanized antibody directed against human C5"* (**D15**, p. 12, 1st par.), in a variant comprising a human IgG4 constant region and a variant comprising a chimeric human IgG2/G4 constant region, the latter of which is termed h5G1.1 CO12 HuG2/G4 (**D15**, p. 12/13, Figure legend of Fig. 15).

250. The h5G1.1 CO12 HuG2/G4 variant is shown not to bind to U937 cells, in contrast to the h5G1.1 CO12 IgG4 variant, indicating that the chimeric constant region reduces interaction of the antibody with the FcRI receptor (**D15**, p. 12, penultimate par., see also Fig. 15).

251. The amino acid sequence of the HuG2/G4 constant region used in **D15** is derivable from, e.g., the sequence of an antibody termed "2A2" (p. 42/43). The amino acid sequences of the variable regions of 2A2 are disclosed in Fig. 9 of **D15**, the full antibody sequence is taught at p. 42/43. The skilled person would thus have no difficulties identifying the constant region sequence within the full antibody sequence disclosed at p. 42/43 of **D15**. This constant region amino acid sequence is identical to the constant region sequence of eculizumab, aside from a short peptide at the C-terminus of the eculizumab heavy chain sequence. This short peptide, however, is included in the sequence of the corresponding expression construct disclosed in **D15** (cf. human G2/G4 expression plasmid insert sequence, p. 44 to 47 of **D15**).

252. As explained above, this chimeric HuG2/G4 constant region was used in a recombinant antibody termed h5G1.1 CO12 HuG2/G4. Taking the disclosure of **D17** into consideration, which teaches that h5G1.1 CO12 comprises the variable regions of the commercial antibody eculizumab, it is clear that the antibody termed "h5G1.1 CO12 HuG2/G4" must be identical in amino acid sequence with the commercial antibody eculizumab.

253. **D15** thus teaches that an antibody comprising the variable regions of the commercial antibody eculizumab in combination with a chimeric HuG2/G4 constant region can be obtained and possesses beneficial properties when compared to a variant comprising an unaltered IgG4 constant region.

254. The combination of **D1** (which refers to **D17**) and **D15** would thus direct the skilled person in an obvious manner to the provision of an alternative antibody consisting of the amino acid sequences defined by SEQ ID NO 2 and residues 23 to 214 of SEQ ID NO:4.

255. Since the problem lies in the provision of an alternative, the skilled person is not required to have a particular motivation to combine documents **D17** and **D15**, it is sufficient that the skilled person could have combined **D1** (together with the documents cited therein) and **D15**.

256. With regard to the feature SEQ ID NO 2 and residues 23 to 214 of SEQ ID NO:4, AR2 therefore lacks an inventive step in view of **D1** in combination with **D15**.

257. The same feature further lacks an inventive step in view of **D1** in combination with (**D17** and) **D43**. As we have noted in our submission dated November 21, 2018, **D43** teaches the (supposedly novel) engineered heavy chain constant region encompassed by SEQ ID NO: 2 of the opposed patent. Specifically, **D43** discloses a recombinant antibody based on the 5G1.1 framework, known from **D17**, wherein the heavy chain CDR3 has been replaced with a clearly defined thrombopoietin mimetic peptide (**D43**, p. 56, Example 4).

258. The full sequence of the resulting antibody, including the engineered heavy chain constant region, is disclosed in Fig. 13A and B of **D43**. Since **D43** refers to **D17** (cf. **D43**, p. 56, li. 4), which discloses the heavy chain CDR3 sequence of eculizumab and **D43** further specifies the peptide that has been used to replace the CDR sequence (cf. **D43**, p. 56, li. 7/8), the skilled person would have had no difficulties to derive the original 5G1.1 sequence from **D43** by replacing the mimetic peptide encompassed by SEQ ID NO: 67 of **D43** by the heavy chain CDR3 sequence of eculizumab disclosed in **D17** (cf. above). This way, the skilled person would have arrived at the amino acid sequence of the eculizumab heavy chain constant region, without having to rely on inventive activity.

259. With regard to the remaining features, AR2 lacks an inventive step due to the reasons provided hereinabove with regard to the Main Request.

IV.2.3 Auxiliary Request 3

IV.2.3.1. Added Matter (Art. 100 (c) EPC / Art. 76 (1) EPC, 123(2) EPC)

260. The alleged basis for the feature *"having a pH of 7.0"* is **WO**, p. 24, 2nd paragraph. This feature is not disclosed in connection with the further features recited in the claim of AR3. It has to be isolated from a list of equivalent features disclosed in the same section.

261. In addition, the pH of 7.0 is not disclosed as an isolated value, but as end point of a range (**WO**, p. 24, li. 11).

262. The isolation of the feature therefore involves a two-fold selection from lists. First, the pH range has to be selected from the numerous equivalent embodiments disclosed in lines 7 to 19 of p. 24 and, second, the end point of the range has to be isolated from the disclosed range of pH values.

263. Due to this two-fold selection, AR3 violates Articles 123(2) and 76(1) EPC.

IV.2.3.2. Inventive Step (Article 100(a) EPC / Article 56 EPC)

264. The Appellant has not provided any arguments why AR3 would overcome the deficiencies of the MR under Article 56 EPC. It fails to do so.

265. The OD has correctly noted with regard to AR1A during opposition proceedings (now AR3) that the specification of the pH value concerns the eculizumab formulation itself and not the method of treatment. It is not clear how the pH of 7.0 would affect the patient and thus be relevant in the context of the original disclosure and the technical problem formulated therein.

266. According to the Appellant, the restriction to a pH of 7.0 is supposed to alleviate the problem of the claims being overly broad (par. 6.4.1 of the statement of grounds). It fails to do so. We refer in this regard to our submission dated November 21, 2018 (section IV.4). It is not credible/plausible, in particular not when considering the Appellant's own considerations regarding the complexity of the formulation process of an antibody, that the technical effect of superior or merely suitable antibody stability over long periods of storage is provided by the concentration of 10 mg/mL over the whole range of conceivable pH values, buffers and salt concentrations.

267. Specification of the pH is insufficient to solve this problem, as critical variables, such as the buffer and the concentration of excipients (such as, e.g., surfactants) are still not defined. The amendment is thus *prima facie* not suitable to overcome the objection.

268. In addition, document **D22** teaches a suitable pH range for the formulations disclosed therein to be from pH 6 to 8 (**D22**, Fig. 10; cf. also par. [0069]). This range includes the pH value specified in AR3. In the absence of any specific technical effect connected to this pH value, the pH of 7.0 thus merely represents an arbitrary choice from equally obvious alternatives.

269. In any case, determining a suitable pH for an antibody formulation to ensure sufficient antibody stability belong to the routine experimentation a formulation scientist would perform in any case. In this regard, review **D20** states:

> *"Formulation development often starts with determination of a stable formulation pH."* (**D20**, p. 15, right col. 1st full par.)

270. A pH in the very middle of the suitable pH range (i.e., pH 6 to 8) for pharmaceutical formulations (cf. **D22**, par. [0069]) would appear to be natural starting point for the skilled artisan.

271. We further note that the reliance on the pH of 7.0 represents an obvious contrast within the Appellant's submission. On the one hand, the third Miano declaration (**D55**) supposedly shows that the purported effect of superior stability is provided regardless of the pH. On the other hand, the specific pH of 7.0 is supposed to serve as a distinguishing feature to form the basis for acknowledging an inventive step.

272. If the data provided by Dr. Miano was accepted to be valid (which we do not), the pH of 7.0 obviously constitutes an arbitrary choice, which does not result in a technical effect that would be connected to the purported technical problem of providing a highly stable, low volume formulation and therefore cannot be taken into consideration when evaluating inventive step.

273. For these reasons, AR3 violates Article 56 EPC.

IV.2.3.3. Lack of sufficient disclosure (Article 100(b) EPC)

274. AR3 lacks sufficient disclosure for the same reasons provided hereinabove for the Main Request.

IV.2.4 Auxiliary Request 4

275. AR4 represent a combination of ARs 1 and 3 and therefore inherits the deficiencies under Articles 123(2) and 76(1) EPC as well as Article 56 EPC. The subject matter of AR4 is neither derivable in a direct and unambiguous manner from the parent or divisional application as filed, nor does it involve an inventive step.

276. Further, AR4 lacks sufficient disclosure due to the same reasons provided for ARs 1 and 3.

IV.2.5 Auxiliary Request 5

277. AR5 is a combination of ARs 2 and 3. Due to the reasons provided hereinabove with regard to ARs 2 and 3, AR5 violates Articles 123(2) and 76(1) EPC, together with Article 56 EPC.

IV.2.6 Auxiliary Request 6

278. AR6 comprises the additional feature (compared to the granted claim) of administration by intravenous infusion. It suffers from at least the same deficiency under Articles 123(2) and 76(1) EPC as the granted claims.

279. The OD has correctly noted that intravenous infusion is disclosed in **D1** (par. [0060]), this additional feature is therefore insufficient to cure any deficiency under Article 56 EPC.

280. The feature of intravenous infusion is further entirely unrelated to the technical problem put forward by the Appellant, i.e., the provision of a highly stable, low volume formulation of eculizumab. The Appellant has thus far not provided any explanation why and how intravenous infusion would contribute to solving the technical problem.

281. The limitation thus represents a randomly isolated feature picked by the Appellant in a desperate attempt to introduce any type of distinguishing feature. Even this is not accomplished, since intravenous infusion is disclosed in the closest prior art.

282. AR6 thus lacks an inventive step due to the same reasons provided supra with regard to the granted claims. Similarly, it violates Articles 123(2) and 76(1) EPC.

283. In addition, AR6 lacks sufficient disclosure due to the same reasons provided for the Main Request.

IV.2.7 Auxiliary Request 7

284. AR7 is a combination of ARs 3 and 6. The above-mentioned applies *mutatis mutandis*.

285. The limitation to a pH of 7.0 or to intravenous administration (alone or in combination) fail to render the subject matter inventive. Notably, the Appellant has not provided any arguments in this regard.

286. Due to the reasons provided *supra* with regard to ARs 3 and 6, AR7 violates Articles 123(2), 76(1) and 56 EPC. Furthermore, AR7 lacks sufficient disclosure based on the same considerations provided hereinabove with regard to ARs 3 and 6.

IV.2.8 Auxiliary Request 8

287. AR8 comprises the additional features of administration via IV infusion and dilution of the composition to a final concentration of 5 mg/mL prior to administration. AR8 lacks sufficient disclosure for the same reasons provided supra with regard to the Main Request.

288. In addition, AR8 contains further violations of the EPC, as will be explained below.

IV.2.8.1. Support (Article 84 EPC)

289. The OD has noted that the fact that the final concentration, i.e., the passage of the original disclosure upon which the introduction of the feature is based, is no longer present in the description of the granted patent is of no relevance when examining the requirements of Article 123(2) EPC (section 3.4 of the decision).

290. It appears that the OD has misinterpreted our argument raised during oral opposition proceedings. Article 84 EPC requires a claim to be clear and concise, as well as being supported by the description.

291. Since the relevant passage has been deleted from the Druckexemplar, the feature may be originally disclosed, however, it is no longer supported by the description.

292. Thus, AR8 violates Article 84 EPC.

IV.2.8.2. Added Matter (Art. 100 (c) EPC / Art. 76 (1) EPC, 123(2) EPC)

293. AR8 suffers from the same deficiencies under Articles 123(2) and 76(1) EPC as the granted claims. In addition, the features introduced into the claim of AR8 are not disclosed in combination with the further features of the claim.

294. For at least these reasons, AR8 violates Articles 123(2) and 76(1) EPC.

IV.2.8.3. Inventive Step (Article 100(a) EPC / Article 56 EPC)

295. As the OD has correctly noted, the feature of IV infusion is disclosed in **D1** and therefore cannot contribute to an inventive step (cf. above).

296. The feature to be taken into account is therefore the final dilution to 5 mg/mL. It is unclear what technical effect would be provided by this dilution step and the Appellant has not provided any arguments in this regard, neither during oral opposition proceedings, nor in the statement of grounds of appeal. The feature thus represents the result of an arbitrary choice and cannot serve as basis for acknowledging an inventive step.

297. The feature is also obviously not connected in any way to the technical problem the concentration of 10 mg/mL relates to (according to the Appellant), namely the provision of a highly stable, low-volume (!) composition.

298. The problem solved (if any) may be formulated as finding a convenient final dilution for the eculizumab composition prior to administration. As correctly concluded by the OD, the skilled person would arrive at the claimed dilution by means of routine experimentation.

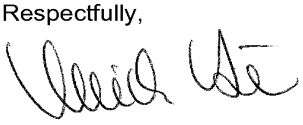
299. In full agreement with the assessment of the OD, we therefore submit that AR8 lacks an inventive step in view of **D1** alone, or in combination with either **D22** or **D45**.

V. Conclusion

300. In summary, the decision of the OD to revoke the patent in its entirety was justified. The Appellant's request to set aside the decision of the OD should therefore be rejected.

301. The Auxiliary Requests submitted by the Appellant fail to cure the deficiencies of the granted claims.

Respectfully,



Dr. H. Ulrich Dörries
European Patent Attorney

UD/SI/KS

Enclosures
Documents **D61** and **D62**

DECLARED at New Haven, CT
United States of America

This 23 Day of May 2017

Bruce A. Andrien Jr.
Bruce A. Andrien Jr., Ph.D.

Witness signature

J. Darrell Fontenot Ph.D., J.D.,
Sr. Dir., Patent Counsel,
Alexion Pharmaceuticals, Inc.
100 College Street, New Haven, CT, 06510,
United States of America

Witness Name, Capacity and Address

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

Exhibit A

BRUCE A. ANDRIEN, Jr., Ph.D.

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CAREER EXPERIENCE:

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

Development responsibilities

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

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

GMP Compliance responsibilities

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

Development responsibilities

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

Other Responsibilities:

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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EDUCATION:

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

FELLOWSHIPS AND HONORS:

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

RELATED ACTIVITIES:

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

PROFESSIONAL SOCIETIES:

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

SPECIAL SKILLS:

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

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SEMINARS:

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

SHORT COURSES:

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

CONFERENCES ATTENDED:

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

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ALEXION TRAINING:

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

Sexual Harassment Prevention Training, (2003)
SOP-QA-0055.00 Labaratory Notebook Requisition, Issuance, and Tracking (22Dec03)
Laboratory Notebook Documentation, Jeff Gassman, (09Dec03)
PowerPoint Microsoft, Key-Logic, Dynamic Slide Enhancements, (28Oct03)
SOP-QCG-0025.03 Quality Control Sample Submission and Handling (20Oct03)
SOP-QA-0017.04 Document Control System, (04Aug03)
Intro to Good Scientific Practices (GSP), (23May03)
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21 CFR Compliance Training Session II; cGMP, GLP, GCP, and QA, (24Jun02)
Pipettes Training, Alpha-Omega, (29May02)
Basic Good Laboratory Practices (21 CFR Part 58), (28May02)

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

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PUBLICATIONS AND PRESENTATIONS:

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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"Performance of an Atmospheric Pressure Corona Discharge Ion Source with Ultrasonic Nebulization", B. A. Andrien, J. P. Quinn, and C. M. Whitehouse, Pittsburgh Conference Proceedings, Chicago (1994).
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"A Comparison of Magnetic Properties of Thin Films of Fe/GaAs(100) and Ni/Mica", B. Andrien and D.R. Miller, Materials Research Society Symposia Proceedings (submitted Dec. 1992). Fall Meeting, Boston, MA, Dec. 1992.

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

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AWARDED PATENTS:

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

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

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

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

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

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

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

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

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

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

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

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PRINCIPLE INVESTIGATOR for the FOLLOWING GRANTS:

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

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

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

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

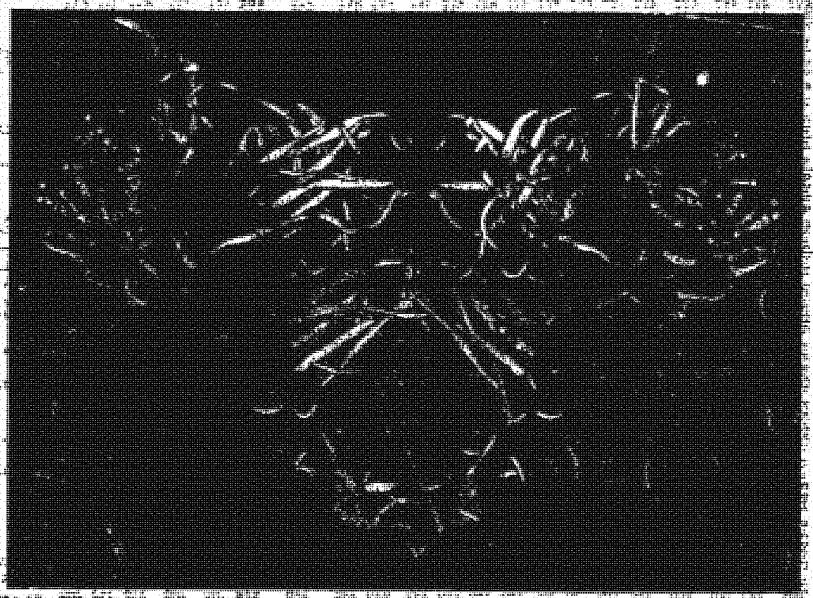
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Exhibit B

SEQUENCES OF PROTEINS OF IMMUNOLOGICAL INTEREST

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FIFTH EDITION



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SEQUENCES OF PROTEINS OF IMMUNOLOGICAL INTEREST

FIFTH EDITION

Tabulation and Analysis of
Amino Acid and Nucleic Acid Sequences of Precursors,
V-Regions, C-Regions, J-Chain, T-Cell Receptors for Antigen,
T-Cell Surface Antigens, β_2-Microglobulins,
Major Histocompatibility Antigens, Thy-1, Complement,
C-Reactive Protein, Thymopoietin, Integrins, Post-gamma Globulin,
α_2-Macroglobulins, and Other Related Proteins

1991

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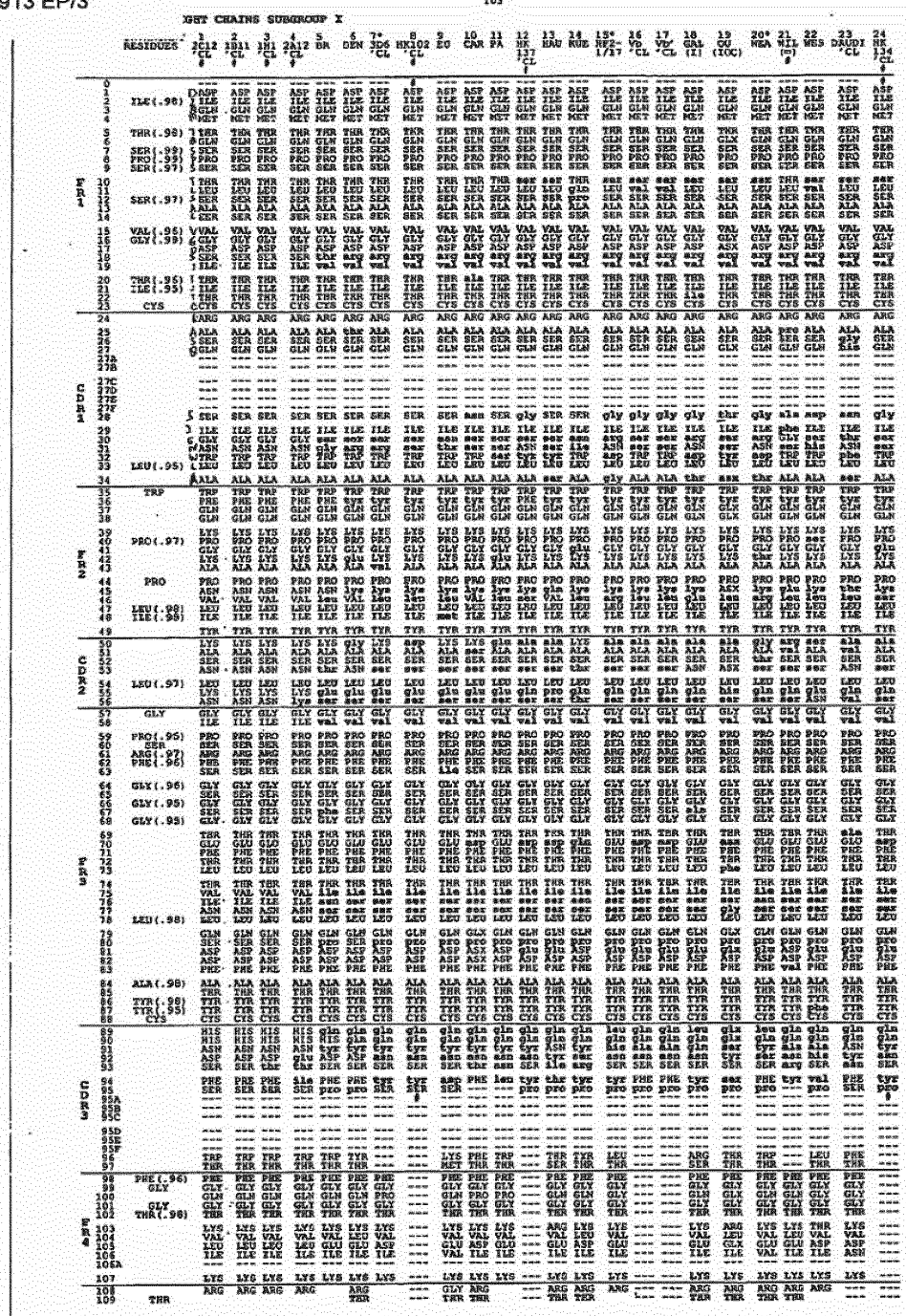
Exhibit D

HUMAN KAPPA LIGHT CHAINS

ANNOTATED RESIDUES	1 HK101 'CL	2 HK 127 'CL	3 VKI-CDR1 'CL	4 HuVH CAMP 'CL	5 VKI-2I 'CL	6 306 'CL	7 V198 'CL	8 Vb 'CL	9 Vb' 'CL	10 HK102 'CL	11 2A12 'CL	12 1R1 'CL	13 2C12 'CL	14 1B11 'CL	15 Vg 'CL	16 Va 'CL	17 Vg 'CL	18 V23 'CL	19 GK 607 'CL	20 A3 'CL	21 RPMI 6410 'CL #	22 AS 'CL	23 A23 'CL
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-22		MET	MET	---	---	THR	MET	MET	MET	MET	MET				MET	MET	---	MET			---	---	---	
-21		ASP	ASP	---	---	ASP	ASP	GLU	ASP	ASP	ASP				ASP	ASP	---	ASP			---	---	---	
-20	MET(.98)	MET	MET	MET	---	VAL	MET	MET	MET	MET	MET				MET	MET	MET	MET		MET	MET	MET	MET	
-19		ARG	ARG	ARG	MET	ARG	ARG	ARG	ARG	MET	ARG	ARG			ARG	ARG	ARG	ARG		ARG	ARG	ARG	ARG	
-18		VAL	VAL	ALA	GLY	ALA	VAL	VAL	VAL	VAL	VAL	VAL			VAL	VAL	VAL	VAL		PRO	LEU	LEU	LEU	
-17		LEU	LEU	PRO	THR	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO		PRO	PRO	PRO	PRO		ALA	PRO	PRO	LEU	
-16		ALA	ALA	THR	GLN	THR	ALA	ALA	ALA	ALA	ALA	ALA	ALA		ALA	ALA	ALA	ALA		ALA	ALA	ALA	ALA	
-15		GLN	GLN	GLN	CYS	GLN	GLN	GLN	GLN	GLN	GLN	LEU	LEU		GLN	GLN	GLN	GLN		GLN	GLN	GLN	GLN	
-14		LEU	LEU	LEU	ILE	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU	
-13		LEU	LEU	LEU	ILE	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU	
-12		GLY	GLY	GLY	LEU	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY		GLY	GLY	GLY	GLY		GLY	GLY	GLY	GLY	
-11		LEU	LEU	LEU	PHE	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU	
-10	LEU	LEU	LEU	LEU	MET	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU	
-9		LEU	LEU	VAL	VAL	GLY	LEU	LEU	LEU	LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU		MET	MET	MET	MET	
-8	LEU(.95)	LEU	LEU	ALA	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU			LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU	
-7	TRP(.95)	CYS	CYS	TRP	THR	TRP	TRP	TRP	THR	THR	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP		VAL	VAL	ILE	VAL	
-6		PHE	PHE	LEU	ALA	LEU	LEU	LEU	PHE	PHE	LEU	LEU	LEU		LEU	LEU	LEU	LEU		VAL	VAL	ILE	VAL	
-5		PRO	PRO	PRO	THR	PRO	PRO	ARG	PRO	PRO	PRO	PRO	PRO		PRO	PRO	PRO	PRO		SER	PRO	PRO	PRO	
-4		GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	
-3		ALA	ALA	ALA	VAL	ALA	ALA	ALA	SER	SER	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	SER	SER	SER	SER	SER	
-2		ARG	ARG	ARG	HIS	ARG	LYS	ARG	ARG	ARG	LYS	LYS	LYS	LYS	ARG	ARG	ARG	ARG	SER	SER	SER	SER	SER	
-1		CYS	CYS	CYS	SER	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	GLY	GLY	GLY	ALA	GLY	

	24 NG9 'CL	25 HIC (RI) 'CL #	26 HAH 14.1 'CL #	27 RAN 14.2 'CL #	28 HAH 16.1 'CL #	29 NOV 'CL #	30 0E10 'CL #	31 TH3 'CL #	32 Humkv 325 'CL	33 EV1-15 'CL	34 IARC/ BL41 'CL	35 Humkv 305 'CL	36 L81 'CL	37 L82 'CL	38 L84 'CL	39 L95 'CL	40 L86 'CL	41 L87 'CL	42 L88 'CL	43 Vg 'CL	44 Vh 'CL	45 CLL 'CL #	46 Hum Ky328 'CL #	47 Hum Kaiser 'CL #
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-20	MET	MET	MET	MET	MET				MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET		
-19	GLU	GLU	GLU	GLU	GLU				GLU	GLU	GLU	GLU	GLU	GLU	GLU	GLU	GLU	GLU	GLU	GLN	GLN	GLU		
-18	THR	THR	THR	THR	THR				THR	THR	THR	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA		
-17	PRO	PRO	PRO	PRO	PRO				PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO		
-16	ALA	ALA	ALA	ALA	ALA				ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA		
-15	GLN	GLN	GLN	GLN	GLN				GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN		
-14	LEU	LEU	LEU	LEU	LEU				LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		
-13	LEU	LEU	LEU	LEU	LEU				LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		
-12	PHE	PHE	PHE	PHE	PHE				PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE		
-11	LEU	LEU	LEU	LEU	LEU				LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		
-10	LEU	LEU	LEU	LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		
-9	LEU	LEU	LEU	LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		
-8	LEU	LEU	LEU	LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		
-7	TRP	TRP	TRP	TRP	TRP	TRP	TRP		TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP		
-6	LEU	LEU	LEU	LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		
-5	PRO	PRO	PRO	PRO	PRO	PRO	PRO		PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO		
-4	VAL	ASP	ASP	ASP	ASP	ASP	ASP		ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP
-3	PRO	THR	THR	THR	THR	THR	THR		THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR
-2	SER	THR	THR	THR	THR	THR	THR		THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR
-1	GLY	GLY	GLY	GLY	GLY	GLY	GLY		GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	ARG	GLY	GLY	GLY

	48 GF4 /J1.1 'CL	49 X- EV15 'CL	50 VKAPPA IV GERMLINE 'CL	51 PBJ7IV 'CL	52 FK-001 'CL #	53 WALKER 'CL	54 DAUDI 'CL	55 VJI 'CL	56 HK100 'CL	57 Va 'CL	58 V2 'CL	59 V14 'CL	60 V12 'CL	61 V55 'CL	62 X- EVJK11 'CL	63 PSEUDO VKAPPA 24'CL	# OF SEQUENCES	# OF AMINO ACIDS
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-20	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET			MET	MET	17	2
-19	GLU	GLY	VAL	VAL	VAL	ARG	ARG	VAL	ARG	ARG	GLN	GLN			GLU	ARG	53	6
-18	ALA	SER	LEU	LEU	LEU	VAL	VAL	LEU	VAL	VAL	VAL	ALA			THR	CYS	53	7
-17	PRO	GLN	GLN	GLN	GLN	PRO	PRO	GLN	PRO	PRO	PRO	PRO			PRO	SER	54	5
-16	ALA	VAL	THR	THR	THR	ALA	ALA	THR	ALA	ALA	THR	THR			ALA	PRO	54	5
-15	MIS	HIS	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN			GLN	HIS	54	4
-14	VAL	LEU	VAL	VAL	VAL	LEU	LEU	VAL	LEU	LEU	LEU	LEU			LEU	PHE	54	4
-13	LEU	LEU	PHE	PHE	PHE	LEU	LEU	PHE	LEU	LEU	LEU	LEU			LEU	LEU	54	3
-12	PHE	SER	ILE	ILE	ILE	GLY	GLY	ILE	GLY	GLY	GLY	GLY	GLY		PHE	GLU	53	6
-11	LEU	PHE	SER	SER	SER	LEU	LEU	SER	LEU	LEU	LEU	LEU	LEU		LEU	LEU	55	5
-10	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		LEU	LEU	58	1
-9	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	VAL	VAL	LEU	PRO	LEU	VAL	58	5
-8	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	SER	LEU	LEU	PHE	58	4
-7	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	59	3
-6	LEU	ILE	ILE	ILE	ILE	LEU	LEU	ILE	LEU	LEU	LEU	LEU	LEU	PHE	LEU	ILE	59	5
-5	PRO	SER	GLN	SER	SER	ARG	ARG	SER	PRO	PRO	PRO	PRO	PRO	PRO	PRO	LEU	59	5
-4	GLU	ASP	GLY	GLY	GLY	GLY	ARG	GLY	GLY	GLY	GLY	GLY	ASP	GLY	ASP	GLU	63	5
-3	THR	THR	ALA	ALA	ALA	VAL	ALA	ALA	ALA	ALA	ALA	ALA	THR	THR	THR	VAL	63	5
-2	SER	ARG	TYR	TYR	TYR	ARG	ARG	TYR	ARG	ARG	ARG	ARG	ARG	ARG	THR	SER	63	6
-1	GLY	ALA	GLY	GLY	GLY	CYS	CYS	GLY	TYR	CYS	CYS	CYS	CYS	SER	GLY	GLY	63	6



HUMAN KAPPA LIGHT CHAINS SUBGROUP I (cont'd)

		25 Vol 'CL	26 WAGNER 'CL	27 KIDD 'CL	28 Va/ 'CL	29 VRI-CDR1 'CL	30 ANGELOID DAN	31 Vo 'CL	32 V13 'CL	33 DEE	34 WIL (-)	35 ANO	36 V100 'CL	37 VRI-EI 'CL	38 REI	39 AU	40 ROY	41 B1	42 NOVN CAMP 'CL	43 AG	44 SCH	45 KEV	46 KA	47 LAY	48 BEL	49 NI
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	1	ASP	ASP	ASP	ala	ASP	ASP	ala	val	ASN	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP
	2	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	val	ILE	ILE	ILE	ILE
	3	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN
	4	leu	MET	MET	leu	MET	leu	MET	MET	MET	MET	MET	val	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	leu	MET
	5	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR
	6	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN
	7	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
	8	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO
	9	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
FR1	10	PHE	SER	SER	SER	SER	SER	SER	SER	SER	THR	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	THR	SER	SER	SER
	11	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU
	12	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
	13	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	VAL	VAL	ALA	ALA
	14	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
	15	VAL	VAL	VAL	VAL	VAL	VAL	VAL	thr	thr	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL
	16	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY
	17	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	gly	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP
	18	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg
	19	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val
	20	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	ile	THR	THR	THR	THR	THR	THR
	21	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	phe	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	leu
	22	THR	THR	THR	THR	THR	THR	THR	ser	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	leu
	23	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS
	24	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	gln	gln	gln	gln	lys	gln	ARG	glu	gln	gln	gln	gln	gln
	25	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ser	ALA	val	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA
	26	SER	SER	arg	SER	SER	SER	SER	SER	gly	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
	27	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN
	27A	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	ser
	27B	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	val
CDR1	27C	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	leu
	27D	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	glu
	27E	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	27F	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	28	gly	SER	gly	gly	gly	SER	gly	gly	SER	SER	SER	gly	gly	asp	asp	asp	asp	asn	asp	asp	SER	thr	asn	asn	asn	SER
	29	ILE	ILE	ILE	ILE	ILE	val	ILE	ILE	val	val	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	asp	val	val	ile	gly	
	30	ser	asn	ser	ser	ser	tyr	ser	ser	asn	ser	GLY	asn	ser	ser	ile	ser	ser	asn	asp	asn	asp	val	leu	asn	ser	asp
	31	ser	ASN	ser	ser	ASN	asn	ser	ser	lys	thr	gly	ASN	ASN	lys	asp	ile	ASN	lys	asn	lys	asp	ser	gln	lys	thr	ser
	32	tyr	tyr	tyr	ala	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr	ser	tyr	tyr	gly	asn	ile	ala	tyr	asp	asp	ser	ile		
	33	LEU	LEU	LEU	LEU	LEU	val	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU
	34	ALA	asn	ALA	ALA	asn	ALA	ALA	ALA	asn	ALA	asn	asn	asn	asn	asn	asn	asn	ile	asn	asn	asn	asn	asn	asn	asn	ALA
	35	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP		TRP
	36	TYR	TYR	TYR	TYR	TYR	PHE	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR		TYR
	37	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	ile	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN		GLN
	38	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN
FR2	39	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS
	40	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO
	41	GLY	GLY	glu	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	lys
	42	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	gln	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS
	43	ALA	ALA	thr	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	thr	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	pro	ALA
	44	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO
	45	lys	lys	lys	lys	lys	lys	lys	glu	lys	ASN	ser	thr	lys	lys	lys	lys	lys	lys	lys	lys	lys	lys	lys	glu	lys	glu
	46	leu	leu	ser	leu	leu	ser	leu	leu	val	leu	leu	leu	leu	leu	leu	phe	leu	leu	leu	leu	leu	leu	leu	leu	leu	leu
	47	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU
	48	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE
	49	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR		TYR	phe	TYR	TYR	TYR	TYR	TYR	TYR	TYR	phe	TYR	TYR	TYR	TYR	TYR		
	50	ala	ala	ala	ser	ala	asp	ala	ala	ala	glu	gly	ala	ala	ser	asp	asp	asp	asp	asp	asp	glu	asp	ala	glu	asp	asp
	51	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA
CDR2	52	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	gln	SER	SER	SER	SER	SER
	53	thr	ser	ser	ser	ser	thr	thr	thr	ser	ile	ser	ser	ser	ASN	ASN	lys	ASN	ASN	ASN	ASN	thr	ASN	ser	thr	thr	ASN
	54	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	arg	LEU	LEU
	55	glu	glu	glu	glu	glu	gln	gln	gln	lys	glu	arg	glu	glu	glu	glu	glu	glu	glu	glu	glu	glu	glu	glu	lys	glu	glu
	56	ile	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser	ala	ile	ser	ala	ile	ser	ser	ala	ser	ser	ser	ser	ser	ile
	57	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY
	58	val	val	val	val	ILE	val	val	val	val	val	val	val	ILE	val	val	val	val	val	val	val	val	val	val	val	val	val
	59	PRO	thr	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO
	60	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
	61	ARG	ARG	ARG	ARG	ARG	asn	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG
	62	PHE	PHE	PHE	SER	PHE	PHE	PHE	PHE	PHE	PHE	THR	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE
	63	SER	SER	SER	SER	SER	thr	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	arg	SER	SER	SER	SER	SER	SER	SER
	64	GLY	GLY	GLY	GLY	asp	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	asp	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY
	65	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	gly	thr	SER	SER	SER	gly	gln	SER	SER	SER	SER	SER
	66	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	ile	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	asp	GLY	GLY	GLY	GLY	GLY	GLY	GLY
	67	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	GLN	SER
	68	GLN	GLY	GLN	GLN	GLY	GLY	GLY	GLY	GLY	GLN	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLN	GLN
	69	THR	THR	THR	THR	ala	THR	THR	THR	THR	THR	THR	THR	ala	THR	ala	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR
	70	GLU	asp	asp	asp	asp	asp	asp	asp	asp	asp	asp	asp	asp	asp	Dis	asp	asp	asp	asp	asp	asp	asp	ala	asp	asp	asp
	71	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE
	72	THR	THR	THR	THR	ile	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	ala	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR
FR3	73	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	phe	phe	phe	LEU	phe	phe	LEU	LEU	phe	phe	LEU	phe	
	74	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	ser	THR	THR	THR	THR	THR	THR	THR	THR	THR
	75	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile
	76	ser	ser	ser	ser	arg	ser	ser	ser	ser	ser	thr	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser
	77	SER	SER	SER	SER	SER	SER	SER	SER	gly	SER	SER	SER	SER	SER	SER	SER	SER	gly	thr	SER	SER	SER	SER	SER	SER	gly
	78	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	val	LEU	LEU	LEU	LEU
	79	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	leu	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN
	80	PRO	PRO	PRO	PRO	PRO	PRO	PRO	SER	SER	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO
	81	glu	glu	glu	glu	glu	glu	glu	glu	glu	ASP	glu	glu	glu	glu	glu	glu	glu	glu	glu	glu	glu	ASP	glu	ala	ala	PRO
	82	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	asn	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP
	83	PHE	ser	PHE	PHE	PHE	PHE	PHE	PHE	PHE	ser	PHE	val	PHE	ile	ile	ile	ile	PHE	ile	ile	ile	PHE	PHE	ile	PHE	PHE
	84	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	gly	ALA	ALA	ALA	ALA	ALA	ALA
	85	THR	THR	THR	THR	THR	THR	THR	THR	THR	arg	ala	THR	ala	THR	THR	THR	THR	THR	THR	ser	THR	THR	THR	THR	val	THR
	86	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR
	87	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	phe	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR
	88	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS
	89	gln	gln	gln	gln	gln	gln	gln	gln	glx	gln	gln	leu	gln	gln	gln	gln	gln	leu	gln	gln	gln	gln	gln	gln	gln	gln
	90	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln
	91	leu	ser	phe	ser	tyr	tyr	ser	tyr	ser	tyr	ser	asp	ser	tyr	tyr	phe	tyr	ile	tyr	tyr	ser	tyr	tyr	ser	tyr	tyr
	92	asn	tyr	asn	asn	asp	asn	tyr	ser	tyr	asn	tyr	ser	tyr	asp	asp	asp	tyr	ile	asp	asp	tyr	leu	asn	asn	asp	asp
	93	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
CDR3	94	tyr	thr	tyr	tyr	thr	tyr	tyr	PHE	thr	tyr	ser	thr	thr	leu	leu	leu	leu	arg	leu	val	asn	leu	trp	PHE	leu	
	95	pro	leu	pro	pro	pro	pro	pro	pro	pro	---	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro
	95A	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	GLU	---	---	---	---	---
	95B	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	95C	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	95D	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	95E	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	95F	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	96	---	ILE	---	---	TYR	---	---	---	TYR	---	TRP	---	---	TYR	TRP	LEU	TYR	ARG	ARG	ILE	VAL	ARG	PRO	LEU	SER	
	97	---	THR	---	---	THR	---	---	---	THR	---	THR	---	---	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	
	98	---	PHE	---	---	PHE	---	---	---	PHE	PHE	PHE	---	---	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	
	99	---	GLY	---	---	GLY	---	---	---	GLY	GLY	GLY	---	---	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	
	100	---	GLN	---	---	GLN	---	---	---	PRO	GLN	GLN	---	---	GLN	GLN	GLY	GLN	GLN	GLN	GLN	GLY	GLN	GLN	GLY	VAL	
	101	---	GLY	---	---	GLY	---	---	---	GLY	GLY	GLY	---	---	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	
	102	---	THR	---	---	THR	---	---	---	THR	THR	THR	---	---	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	
FR4	103	---	ARG	---	---	LYS	---	---	---	LYS	LYS	LYS	---	---	LYS	LYS	LYS	LYS	LYS	LYS	ARG	THR	LYS	LYS	GLU	LYS	
	104	---	LEU	---	---	---	---	---	---	VAL	LEU	VAL	---	---	VAL	VAL	VAL	LEU	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	
	105	---	GLU	---	---	---	---	---	---	GLU	GLU	GLN	---	---	GLN	GLU	ASP	GLU	GLU	GLU	GLU	ASP	ASP	GLU	GLU	GLU	
	106	---	ILE	---	---	ILE	---	---	---	MET	ILE	ILE	---	---	ILE	ILE	PHE	ILE	ILE	ILE	ASN	ILE	LEU	VAL	VAL	VAL	
	106A	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
	107	---	LYS	---	---	---	LYS	---	---	THR	LYS	ARG			THR	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS
	108	---	---	---	---	---	ARG	---	---	ARG	ARG				ARG	ARG	ARG	ARG	ARG	ARG	GLY	ARG	ARG	ARG		ARG	
	109	---	---	---	---	---	---	---	---	THR	THR				THR	THR	THR	THR		THR	THR		THR			THR	

HUMAN KAPPA LIGHT CHAINS SUBGROUP I (cont'd)

		50 RE	51* Ku RSV19 VK #	52 HBJ 4	53 BJ 26 f	54 BJ 19 #	55 RFZ	56 HA	57 PAU	58 FSH	59 JBL #	60 NE	61 FRA	62 BJ 48	63 MF3- 16/6	64 MF2- 1/13B	65 MF24 10/2	66 GR'	67 GL 'CL #'	68 HOM	69 KER	70 ESM IGC	71 ESH IGH	72* DAV	73* FIN	74 PM #	75 BRE	76 ADA
	0	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---		---	---	---	---	---	---	---	---	---
	1	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP		ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP
	2	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE		ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE
	3	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	his	GLN	GLN	GLN	GLN	GLN	GLN		GLN	GLN	GLN	GLN	GLN	GLN	his	GLN	GLN
	4	MET	met	MET	MET	MET	MET	MET	MET	MET	MET	leu	MET	MET	MET	MET	MET	MET		MET	MET	MET	MET	MET	MET	leu	val	
	5	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR		THR	THR	THR	THR	THR	THR	THR	THR	THR
	6	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLX	GLN	GLN	GLN	GLN	GLN	GLN		GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN
	7	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER		SER	SER	SER	SER	SER	SER	SER	SER	SER
	8	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO		PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO
	9	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER		SER	SER	SER	SER	SER	SER	thr	SER	
F	10	ser	ser	THR	ser	ser	ser	ser	THR	ser	ser	ser	ser	THR	ser	ser	ser	THR		ser	ser	ser	ser	ser	ser	ser	ser	ser
R	11	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU
1	12	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER		SER	SER	SER	SER	SER	SER	SER	SER	SER
	13	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA		ALA	ALA	ALA	ALA	thr	thr	ALA	ALA	val
	14	SER	SER	SER	SER	SER	SER	SER	SER	SER	tyr	SER	SER	SER	SER	SER	SER	SER		SER	SER	SER	SER	val	val	SER	SER	phe
	15	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL		VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL
	16	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY		GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY
	17	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP		ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASX	ASP
	18	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg		arg	arg	arg	arg	arg	arg	arg	arg	arg
	19	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val		val	ile	val	val	val	val	val	val	val
	20	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR		THR	THR	THR	THR	THR	THR	THR	THR	THR
	21	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE		ILE	ILE	ILE	ILE	ILE	val	phe	ILE	
	22	THR	THR	THR	THR	THR	THR	THR	THR	THR	ala	THR	THR	THR	THR	THR	THR	THR		THR	THR	THR	THR	THR	arg	THR	THR	
	23	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS		CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	
	24	gln	ARG	ARG	gln	gln	gln	gln	ARG	gln	ARG	glx	ARG		ARG	ARG	ARG	ARG		gln	gln	ARG	ARG	asp	asp	ARG	gln	gln
	25	ALA	ser	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA		ALA	ALA	ALA	ALA		ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA
	26	asn	SER	SER	SER	SER	asn	SER	SER	SER	SER	SER	SER		SER	SER	SER	SER		arg	SER	SER	SER	SER	SER	SER	SER	SER
	27	GLN	GLN	GLN	GLN	GLN	GLN	---	GLN	GLN	GLN	GLN	GLX		GLN	GLN	GLN	arg		ala	GLN	GLN	GLX	GLN	GLN	GLN	GLX	GLX
	27A	---	thr	---	---	---	---	---	---	---	---	---	---		---	---	---	---		---	---	---	---	---	---	---	---	---
	27B	---	leu	---	---	---	---	---	---	---	---	---	---		---	---	---	---		---	---	---	---	---	---	---	---	---
	27C	---	val	---	---	---	---	---	---	---	---	---	---		---	---	---	---		---	---	---	---	---	---	---	---	---
C	27D	---	his	---	---	---	---	---	---	---	---	---	---		---	---	---	---		---	---	---	---	---	---	---	---	---
D	27E	---	thr	---	---	---	---	---	---	---	---	---	---		---	---	---	---		---	---	---	---	---	---	---	---	---
R	27F	---	---	---	---	---	---	---	---	---	---	---	---		---	---	---	---		---	---	---	---	---	---	---	---	---
1	28	val	asp	SER	SER	asp	val	---	SER	asp	SER	asn	asn		gly	gly	gly	SER		asp	asp	SER	SER	asn	asn	SER	asn	asn
	29	ILE	gly	val	ILE	ILE	ILE	---	ILE	ILE	ILE	ILE	ILE		ILE	ILE	ILE	ILE		ser	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE
	30	ser	asn	asn	asn	asn	ser	arg	ser	arg	ser	ser	asn		asp	arg	arg	ser		ser	lys	ser	ser	ser	asx	GLY	ser	
	31	lys	thr	ASN	lys	thr	lys	ASN	asr	ser	thr	ASX	ASX		ASN	ASN	ASN	asr		ASN		ser	ser	ser	ser		ASX	ASX
	32	ser	tyr	TRP	tyr	phe	ser	tyr	ser	tyr	phe	tyr	tyr		asp	asp	asp	tyr		tyr		tyr	tyr	TRP	TRP	tyr	tyr	
	33	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU		LEU		LEU	LEU	LEU	LEU	ser	LEU	LEU
	34	asn	glu	ALA	asn	asn	asn	asn	ALA	asn	#	asx	ALA		gly	gly	gly	ALA		asn		asx	asx	ile	ile	asx	asx	asx
	35	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP		TRP	TRP	TRP	TRP		TRP		TRP	TRP	TRP	TRP	TRP	TRP	TRP
	36	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr		tyr	tyr	tyr	tyr		phe		tyr	tyr	tyr	tyr	tyr	tyr	tyr
	37	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLX	GLN	GLX		GLN	GLU	GLN	GLN		GLN		GLX	GLX	GLN	GLN	GLN	his	GLX
	38	GLN	GLN	gln	GLN	GLN	GLN	GLN	GLX	GLN	GLN	GLX	GLX		GLN	GLN	GLN	gly		GLN				GLN	GLN	GLN	GLX	tyr
	39	arg	LYS	LYS	#	#	arg	LYS	LYS	LYS	LYS	LYS	LYS		LYS	LYS	LYS	arg		LYS				tyr	tyr	LYS	LYS	LYS
	40	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	gln	PRO	PRO		PRO	PRO	PRO	PRO		PRO				PRO	PRO		PRO	PRO
	41	GLY	GLY	GLY	#	#	GLY	GLY	GLY	GLY	GLY	GLY	GLY							GLY								
H	42	gln	LYS	gln	LYS	LYS	glu	arg	glx	LYS	LYS	glx																
C	43	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA		ALA														
D	44	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO		PRO														
R	45	lys	lys	lys	lys	lys	lys	lys	lys	lys	lys	gly		lys														
2	46	ile	ile	ile	leu	leu	leu	leu	leu	leu	leu	VAL																
	47	LEU	LEU	LEU			LEU	LEU	LEU	LEU	LEU	LEU		met														
	48	ILE	ILE	ILE			ILE	ILE	ILE	ILE	ILE			ILE														
	49	TYR	TYR	ser			TYR	TYR	TYR	TYR	TYR	TYR	TYR		gly													
	50	asp	asp	LYS			asp	asp	asp	glx	asp				asp													
C	51	ALA	val	thr	asp	ALA	ALA	ALA			ALA				ALA													
D	52	SER	SER	SER	SER	SER	ala	phe			SER				SER													
R	53	ASN	ASN	ser	arg	ser	ASX	ASN							ser													
2	54	LEU	arg	LEU	LEU	LEU	LEU	LEU			LEU				LEU													
	55	glu	phe	glu	glu	glu	glu	glu			glu				asn													
	56	ser	ser	arg	thr	thr		ala							ser													
	57	GLY	GLY	GLY	GLY	GLY	GLY	GLY							GLY													
	58	val	val	val	val	asn	val	val							val				ILE									
	59	PRO	PRO	PRO	PRO	glx	PRO	PRO							val				PRO									
	60	SER	SER	SER	SER	SER		SER							SER				SER									
	61	ARG	ARG	ARG	lys	ARG	ARG	ARG							ARG				ARG									
	62	PHE	PHE	PHE	val	PHE	PHE								glu				PHE									
	63	thr	SER	ala	SER	leu	thr								SER				SER									
	64	GLY	GLY	GLY	GLY	GLY	GLY								GLY				GLY									
	65	SER	SER	SER	SER	SER									SER				SER									
	66	GLY	GLY	GLY	asn	GLY									GLY				GLY									
	67	SER	SER	SER	phe										SER				SER									
	68	GLY	GLY	glu	asn										GLY				GLY									
	69	THR	THR	THR	THR										THR				THR									
	70	asp	asp	asp	lys										asp				GLU									
	71	phe	TYR	phe											pro				PHE									
F	72	THR	THR	THR											ser				THR									
R	73	LEU	phe	val											LEU				LEU									
3	74	THR	THR	ala											ala				THR									
	75	ile	ile	gln											VAL				VAL									
	76	ser	ser	ser											ser				ILE									
	77	ser	ser	ser											arg				ASN									
	78	leu	LEU	LEU															LEU									
	79	arg	GLN	GLN															GLN									
	80	pro	PRO	PRO															SER									
	81	asn	GLN	GLN															ASP									
	82	ASP	ASP	ASP															ASP									
	83	val	ile	ile															PHE									
	84	ALA	ALA	ALA															ALA									
	85	THR	THR	pro															THR									
	86	TYR	TYR	lys															TYR		TYR							
	87	phe	TYR																TYR		TYR							
	88	CYS	CYS																CYS		CYS							
	89	gln	phe		gln														HIS		gln							
	90	glu	gln		gln														HIS		gln							
	91	tyr	gly		arg	phe									gly				ASN		tyr							
	92	ala	ser		LEU	ASP									glu				ASP		ASP							
	93	asn	his		arg	arg									ser				thr		asp							
C	94	leu	leu		leu	leu									asp				PHE		leu							
D	95	pro	pro		pro	pro									SER				SER		pro							
R	95A	---	---		---	---									---				---		---							
3	95B	---	---		---	---									---				---		---							
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	95F	---	---		---	---									---				---		---							
	96	LEU	ARG		TYR	TYR									ARG				TRP		PRO							
	97	THR	THR		THR	THR									THR				THR		THR							
	98	PRO	PRO		LEU	PRO									PRO				PRO		PRO							
	99	GLY	GLY		GLY	GLY									GLY				GLY		GLY							
	100	GLY	GLY		GLY	PRO									GLN				GLN		PRO							
	101	GLY	GLY		GLY	GLY									GLY				GLY		GLY							
	102	THR	THR		THR	THR									THR				THR		THR							
J	103	LYS	LYS		LYS	LYS									LYS				LYS		LYS							
R	104	VAL	VAL		VAL	VAL									VAL				VAL		VAL							
4	105	GLU	GLU		GLU	GLU									GLU				LEU		ASP							
	106	ILE	ILE		MET	LEU									VAL				ILE		LEU							
	106A	---			---	---									---				---		---							
	107	LYS			LYS	LYS									LYS				LYS		LYS							
	108																		ARG									
	109																											

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HUMAN KAPPA LIGHT CHAINS SUBGROUP I (cont'd)

	77 AMYLOID VIII-S	78 PAUL	79 RI #	80 HAT	81* LOH	82 AMYLOID ES305 #	83 OCO	84 HOL	85 AMYLOID X	86 SAC	87 BJ	88 GO	89 F-GUI #	90 S-GUI #	91 HPG-21/28	92 DIE	93 CAR A	94 TEI	95 MON	96 ALE	97 JOH	98 POT	99 COH	100 TRA	101* R-G2b
0	---	---	Pro	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
1	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASX	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASX	ASP	ASP	ASP	ASP	ASP
2	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE		VAL	ILE	ILE	VAL	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE
3	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN		GLN	GLN	GLN	VAL	GLN	GLN	GLN	GLN	GLX	GLN	GLN	GLN	GLN	GLN
4	MET	MET	MET	MET	MET	leu	MET	ile	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET
5	THR	THR	THR	THR	THR	THR	THR	met	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR
6	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLX	GLN	GLN	GLN	GLX	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLX	GLN	GLN	GLN	GLN	GLN
7	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER		SER	SER	ala	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
8	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	ala	PRO		PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO
9	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER		SER	SER	SER	leu	SER	SER	SER	SER	SER	SER	ala	SER	SER	SER
FR 1 10	---	THR	THR	---	---	---	---	---	---	---		pro	---	---	---	---	---	---	THR		---	---	---	---	---
11	LEU	LEU	LEU	LEU	LEU	LEU	LEU	val	LEU	LEU		val	LEU	LEU	LEU	LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU	LEU
12	SER	SER	SER	SER	SER	SER	VAL	SER	SER	SER		SER	SER	SER	pro	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
13	ALA	ALA	ALA	ALA	ALA	ALA	---	ala	ALA	ALA		ALA	ALA	ALA	val	ALA	ALA	ALA	ALA	SER	ALA	ALA	ALA	ALA	ALA
14	SER	SER	SER	SER	SER	SER	pro	SER	SER	SER		SER	SER	SER	thr	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
15	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL		VAL	VAL	VAL	leu	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL
16	GLY	GLY	GLY	GLY	GLY	GLN	GLY	GLY	GLY	GLY		GLY	GLY	GLY	GLN	GLN	GLN	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY
17	ASP	ASP	ASP	ASP	ASP	ASP	ASX	ASX	ASP	ASP		ASX	ASX	ASX	gly	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP
18	asy	asy	gly	asy	asy	---	asy	asy	asy	---	asy	asy	asy	asy	pro	asy	asy	asy	asy	asy	asy	asy	asy	asy	asy
19	val	val	val	val	val	val	val	val	val	---	val	val	val	val	ile	val	val	val	val	val	val	val	val	val	val
20	THR	THR	THR	THR	THR	THR	THR	THR	ile	---	THR	THR	THR	THR	ser	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR
21	ILE	ILE	ILE	ILE	ILE	?	ILE	ILE	ILE	---	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	leu	ILE	ILE	ILE	ILE	ILE
22	THR	THR	ser	THR	THR	THR	THR	THR		---	THR	THR	THR	THR	ser	THR	THR	THR	THR	ser	THR	THR	THR	THR	THR
23	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	---	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS		CYS	CYS	CYS			
24	gln	ARG	ARG	glx	ala	gln	glx	ARG	glx	---	gln	ARG		ARG	ARG					ARG					
25	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	---	ALA	ALA	ALA	ALA	ser										
26	SER	SER	SER	SER	SER	SER	SER	SER		---	SER	SER			SER										
27	GLN	GLN	GLX	GLN	GLN	glu	GLX	GLX	GLX	---	GLN				GLN										
27A	---	---	---	---	---	---	---	---	---	---	---				ser										
27B	---	---	---	---	---	---	---	---	---	---	---				Asn										
27C	---	---	---	---	---	---	---	---	---	---	---				val										
CDR 27D	---	---	---	---	---	---	---	---	---	---	---				Gly										
27E	---	---	---	---	---	---	---	---	---	---	---				ser										
27F	---	---	---	---	---	---	---	---	---	---	---				---										
1 28	asx	SER	SER	asp	asx	ala	asn	gly	asx	---	asp				asp										
29	ILE	ILE	ILE	ILE	ILE	?	ILE	ILE	ILE	---	ILE				gly										
30	GLY	ser	ser	ser	ser		ser			---	ser				ser										
31		ser		asp		ASN	ASX		pro	---	pro				ser										
32	thr	ser		thr		thr	thr		thr	---					Leu										
33	LEU			val		LEU			LEU	---					LEU										
34				asp						---					glu										
35	THR			THP		THR				---					THR										
36						thr			thr	---					thr										
37										---					GLN										
38										---					GLN										
39										---					LYS										
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87										---	TYR														
88										CYS	CYS														
89										glx	glx														
90										GLN	gln														
91										ala	tyr														
92										ASX	GLN														
93										ala	asn														
CDR H3 94										ala	leu														
95										thr	pro														
95A										---	---														
95B										---	---														
95C										---	---														
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97										PRO															
98										ILE															
99										GLY															
100										GLY															
101										GLY															
102										THR															
FR 4 103										LYS															
104										VAL															
105										ASX															
106										VAL															
106A										---															
107										LYS															
108										ARG															
109										THR															

HUMAN KAPPA LIGHT CHAINS SUBGROUP I (cont'd)

		102+ R-G2	103 ZIKA	104 LOK	105* HIL	106 PAP	107* R-G2a	108 CHA	109 AMYLOID LEP	110* MAG	111 NOE	112 RBJ 1	113 AMYLOID 547	114 WEB	115 RBJ 10	116 LEO	117 BEN	118 MAR	119 GR	120 MUR	121 GM131 'CL 6	122 AMYLOID 984	123* MAR	124 CL*
	0	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---			---	---
	1	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP		ASP	ASP	---
	2	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	VLN	ILE	ILE	ILE		ILE	ILE	---
	3	GLN	GLN	GLN	GLN	GLN		GLU	GLN	GLN	GLN	leu	GLX	GLN	GLX	GLN	GLN	GLN	GLN	GLN		GLN	GLN	---
	4	MET	MET	leu	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET		MET	leu	MET	MET		MET	leu	---
	5	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR		THR	THR	---
	6	GLN	GLX	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLX	GLN	GLN	GLN	GLN	GLN	GLN	GLN		GLN	GLN	---
	7	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER		SER	SER	---
	8	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO		PRO	PRO	---
	9	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	thr	SER	SER	SER	SER	SER	SER	SER	SER		SER	SER	---
FR1	10	ser	ser	phe	THR	ser	ser	ser	ser	ser	THR	ser	ser	ser	ser	THR	ser	THR	ser	ser		ser	ser	---
	11	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	val	LEU		LEU	LEU	LEU	LEU		LEU	LEU	---
	12	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER				---
	13	ALA	val	ALA	ALA	val	ALA	ALA	ALA	ALA	SER	SER	ALA	ALA	ALA	ALA	ALA	ALA	val					---
	14	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER									---
	15	VAL	VAL	VAL	VAL	VAL	VAL	leu	VAL	VAL	VAL	VAL	VAL	VAL		VAL								---
	16	GLY	GLY	GLY	GLY	GLY	GLY	asp	GLY	GLY		GLY												---
	17	ASP	glu	ASP	ASP	ASP	ASP	ASP	ASP	ASP		ASP												---
	18	arg	arg	arg	arg	arg	arg	arg	arg															---
	19	val	val	val	val	val	val	val	val															---
	20	THR	THR	THR	THR	THR		THR	THR															---
	21	ILE	ILE	ILE		ILE	ILE	ILE																---
	22	THR	THR	THR		ala	SER	THR																---
	23		CYS																					---
	24																							---
	25																							---
	26																							---
	27																							---
	27A																							---
	27B																							---
	27C																							---
CDR1	27D																							---
	27E																							---
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FR2	42																							---
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CDR2	53																							---
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FR3	73																							---
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	77																							---
	78																				LEU			---
	79																				GLN			---
	80																				PRO			---
	81																				GLU			---
	82																				ASP			---
	83																				PHE			---
	84																				ALA			---
	85																				THR			---
	86																				TYR			---
	87																				TYR			---
	88																				CYS			---
	89																				gln			---
	90																				glu			---
	91																				ala			---
	92																				ASP		ASP	---
	93																				ile		ile	---
	94																				leu		PHE	---
	95																				pro		pro	---
CDR3	95A																				---		---	---
	95B																				---		---	---
	95C																				---		---	---
	95D																				---		---	---
	95E																				---		---	---
	95F																				---		---	---
	96																				LEU		GLY	---
	97																				THR		THR	---
	98																				PHE		PHE	---
	99																				GLY		GLY	---
	100																				GLY		GLN	---
	101																				GLY		GLY	---
	102																				THR		THR	---
FR4	103																				THR		LYS	---
	104																				VAL		VAL	---
	105																				GLU		GLU	---
	106																				MET		ILE	---
	106A																				---		---	---
	107																				LYS		LYS	---
	108																				ARG		ARG	---
	109																				THR		THR	---

HUMAN KAPPA LIGHT CHAINS SUBGROUP I (cont'd)

		125 AMYLOID	126 SEQ .6	127 AMYLOID MS #	128 PEN	# OF SEQUENCES	# OF AMINO ACIDS	OCCURRENCES OF MOST COMMON AMINO ACID	VARIABILITY†
	0	---	---	---	---	1	1	1 (FCA)	
	1	ASP	ASP	ASP	ASP	125	3,4	122 (ASP), 118 (ASP)	3.1,4.2
	2	ILE	ILE	#	ILE	123	2	120 (ILE)	2.
	3	glu	GLN	GLN	GLN	123	7	116 (GLN), 112 (GLN)	7.4,7.7
	4	MET	MET	MET	MET	124	4	109 (MET)	4.6
	5	PRO	THR	THR		124	3	122 (THR)	3.
	6	GLN	GLN			123	1,2	123 (GLN), 116 (GLN)	1.,2.1
	7	SER				121	2	120 (SER)	2.
	8	PRO				121	2	120 (PRO)	2.
	9	SER				121	4	117 (SER)	4.1
FR 1	10	###				120	5	89 (SER)	6.7
	11	LEU				119	4	111 (LEU)	4.5
	12					118	3	113 (SER)	3.1
	13					117	4	107 (ALA)	4.4
	14					119	6	106 (SER)	6.4
	15					113	3	109 (VAL)	3.1
	16					109	5	108 (GLY)	5.
	17					109	3,4	106 (ASP), 98 (ASP)	3.1,4.4
	18					106	6	97 (ARG)	6.6
	19					107	3	101 (VAL)	3.2
	20					106	4	102 (THR)	4.2
	21					104	4	99 (ILE)	4.2
	22					104	6	95 (THR)	6.6
	23					96	1	96 (CYS)	1.
	24					88	6	57 (ARG)	9.3
	25					68	6	82 (ALA)	6.4
	26					85	4	79 (GLN)	4.3
	27					84	4	79 (GLN), 66 (GLN)	4.3,5.1
	27A					3	2	2 (SER)	
	27B					3	2	2 (LEU)	
CDR 1	27C					3	2	2 (VAL)	
	27D					3	3	1 (+)	
	27E					2	2	1 (+)	
	27F								
	28					84	7	29 (SER)	20.
	29					83	5	72 (ILE)	5.8
	30					81	11	41 (SER)	22.
	31					48	12	27 (ASN), 24 (SER)	35.,39.
	32					79	8	34 (TYR)	21.
	33					77	4	73 (LEU)	4.2
	34					73	7,8	30 (ALA)	17.,19.
	35					76	1	76 (TRP)	1.
	36					74	2	66 (TYR)	2.2
	37					73	4	68 (GLN), 61 (GLN)	4.3,4.8
	38					71	4	68 (GLN), 65 (GLN)	4.2,6.5
	39					68	4	62 (LYS)	4.6
	40					70	2	68 (PRO)	3.1
FR 2	41					68	3	56 (GLY)	3.4
	42					60	6	49 (LYS)	7.2
	43					61	4	57 (ALA)	4.5
	44					61	1	61 (PRO)	1.
	45					61	7,8	46 (LYS)	9.3,11.
	46					60	2	42 (LEU)	10.
	47					59	2	58 (LEU)	2.
	48					57	2	55 (ILE)	2.
	49					59	4	55 (TYR)	4.3
	50					59	3	33 (ALA)	28.
	51					59	2	50 (ALA)	5.8
CDR 2	52					59	6	53 (SER)	6.6
	53					57	6,7	26 (SER)	13.,15.
	54					58	2	56 (LEU)	2.1
	55					58	8,9	25 (GLU)	17.,21.
	56					56	8	34 (SER)	13.
	57					57	1	57 (GLY)	1.
	58					58	3	50 (VAL)	3.5
	59					57	4	54 (PRO)	4.2
	60					57	2	57 (SER)	2.
	61					58	3	56 (ARG)	3.1
	62					57	3	55 (PHE)	3.1
	63					57	6	50 (SER)	6.8
	64					57	2	55 (GLY)	2.1
	65					56	5	51 (SER)	5.5
	66					57	4	54 (GLY)	4.2
	67					55	3	52 (SER)	3.2
	68					55	3	52 (GLY)	3.2
	69					55	2	51 (THR)	2.2
	70					55	5,6	33 (ASP), 31 (ASP)	8.3,11.
	71					54	4	49 (PHE)	4.4
FR 3	72					54	5	50 (THR)	5.4
	73					54	3	43 (LEU)	3.8
	74					54	4	51 (THR)	4.2
	75					54	3	47 (ILE)	3.4
	76					53	5	43 (SER)	6.2
	77					54	6	40 (SER)	8.1
	78					54	2	53 (LEU)	2.
	79					54	3,4	52 (GLN), 48 (GLN)	3.1,4.5
	80					54	2	46 (PRO)	2.5
	81					54	3,5	33 (GLU), 24 (GLU)	4.4,7.9
	82					54	2	53 (ASP), 50 (ASP)	2.,2.2
	83					54	4	40 (PHE)	5.4
	84					54	2	53 (ALA)	2.
	85					54	6	48 (THR)	8.7
	86					56	2	55 (TYR)	2.
	87					55	2	52 (TYR)	2.1
	88					56	1	56 (CYS)	1.
	89					57	4,5	46 (GLN), 43 (GLN)	5.,6.5
	90					57	2,3	51 (GLN), 46 (GLN)	2.2,3.7
	91					58	11,12	20 (TYR)	23.,23.
	92					60	5	20 (ASN), 19 (+)	27.,28.
	93					60	9	29 (SER)	19.
CDR 3	94					60	12	15 (LEU)	48.
	95					56	4	44 (PRO)	5.1
	95A					2	1	1 (GLU)	
	95B								
	95C								
	95D								
	95E								
	95F								
	96					44	12	10 (TRP)	53.
	97					43	6	39 (THR)	4.4
	98					46	3	44 (PHE)	3.1
	99					46	1	46 (GLY)	1.
	100					46	4,5	28 (GLN), 27 (GLN)	8.6,8.5
	101					46	1	46 (GLY)	1.
	102					46	2	45 (THR)	2.
FR 4	103					46	4	38 (LYS)	4.8
	104					45	2	36 (VAL)	2.5
	105					45	4,5	29 (GLU), 28 (GLU)	6.2,8.
	106					46	7	30 (ILE)	11.
	106A								
	107					45	3	42 (LYS)	3.2
	108					23	2	21 (ARG)	2.1
	109					22	1	22 (PRO)	1.

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

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).

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

Docket No.: AXJ-114BUSCN2
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

Application No.: 15/148,839

Confirmation No.: 3159

Filed: May 6, 2016

Art Unit: 1644

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

Examiner: J. L. Rogers

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

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

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

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

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

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

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

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

Samsung Bioepis Exhibit No. 1002
Page 13880

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

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

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

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

Samsung Bioepis Exhibit No. 1002
Page 13881

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

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

Samsung Bioepis Exhibit No. 1002
Page 13882

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

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

> "THE INVESTIGATOR AND ALL EMPLOYEES AND COWORKERS INVOLVED WITH THIS STUDY SHALL NOT DISCLOSE OR USE FOR ANY PURPOSE, OTHER THAN PERFORMANCE OF THE STUDY, ANY DATA, RECORDS OR OTHER UNPUBLISHED, CONFIDENTIAL INFORMATION DISCLOSED TO THOSE INDIVIDUALS FOR THE PURPOSE OF THE STUDY. PRIOR WRITTEN AGREEMENT FROM THE SPONSOR OR ITS DESIGNEE MUST BE OBTAINED FOR THE DISCLOSURE OF ANY SAID CONFIDENTIAL INFORMATION TO OTHER PARTIES."

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

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

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

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

> "Eculizumab must be stored in a secure, limited-access storage area. . . .
>
> Test Article Accountability…When a drug shipment is received at the site, the pharmacist at each site shall verify the contents, sign the packing invoice provided with the shipment, and maintain the original copy for review by the study monitor. The duplicate copy should be kept in the pharmacy binder.
>
> Each shipment of investigational drug supplies will contain an accountability log to assist the pharmacist in maintaining current and accurate inventory records covering receipt, dispensing, and disposition of the investigational product. During the study, the following information must be noted in the accountability log: the identification number(s), initials of patient(s) who is dispensed investigational product, the date(s) that the investigational product is prepared and dispensed, and the initials of the pharmacist or designee who prepared the investigational product. Empty vials and vials with residual materials should be kept for inspection by the study monitor.

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

11. All of the protocols that I reviewed for the studies listed in the above table included similar (if not identical) drug storage and accountability provisions, except for the PNH Registry protocol (the last entry in the table above). The PNH Registry was not a study in which eculizumab was administered to any patients, but was a registry for all participating patients with PNH either treated or untreated. More particularly, it was a "non-interventional study designed to collect post-marketing pharmacovigilance and effectiveness data on patients treated with Soliris® (eculizumab)" and to "collect information on the progression of disease in the Soliris treated and untreated PNH patient population." Accordingly, the protocol for the PNH Registry did not contain drug storage or accountability provisions. The PNH Registry protocol did, however, include strict confidentiality provisions as indicated in the table above, *e.g.*, requiring all physicians to "agree to maintain the confidentiality of all information received or developed in connection with [the] protocol."

12. To participate in each clinical trial listed in the table above, each physician had to sign the protocol for the trial, committing to adhere to the respective clinical trial protocol's provisions. For example, the TRIUMPH study protocol, just prior to the physician's signature line, states:

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

13. All of the other protocols that I reviewed similarly required each physician (or investigator) to agree to conduct the study in accordance with the protocol.

14. In response to the Examiner's Request (G), based on the BLA approval letter that I reviewed from the Food and Drug Administration Department of Health & Human Services to Alexion regarding eculizumab, the antibody having the heavy chain described by SEQ ID NO: 2 and the light chain described by SEQ ID NO: 4 was first approved for commercial marketing and sale on March 16, 2007.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or of any patent issued thereon.

Laural S. Boone 11 MAY 2017

Laural S. Boone, J.D., Ph.D. Date

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: January 19, 2017
Electronic Signature for Jill Gorny Sloper, Esq.: /Jill Gorny Sloper, Esq./

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

Docket No.: AXJ-114BUSCN3
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

Application No.: 15/260,888

Filed: September 9, 2016

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

Confirmation No.: 2558

Art Unit: 1644

Examiner: J. L. Rogers

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

DECLARATION PURSUANT TO 37 CFR 1.132 BY DR. LEONARD BELL

I, Leonard Bell, M.D., a co-inventor of the subject invention, declare that:

1. I founded Alexion Pharmaceuticals, Inc. and served as its Chief Executive Officer from 1992 until 2015. In that role, I was closely involved in the development of eculizumab and in the administration and monitoring of all clinical trials involving eculizumab.

2. Prior to March 15, 2007, which is the date the subject patent application was filed, eculizumab was not available to the public. In order to be approved for marketing, eculizumab was used in clinical trials before March 15, 2007, but only under strict control and conditions of confidentiality. Patients in clinical trials of eculizumab received intravenous infusions of eculizumab at hospitals and infusion clinics and never themselves possessed samples of eculizumab. Researchers and staff involved in clinical trials of eculizumab were not permitted to use or distribute eculizumab for

purposes other than conducting the clinical trials, and were strictly accountable to Alexion for the inventory of eculizumab and for information about eculizumab.

3. Moreover, prior to March 15, 2007, the complete structure of eculizumab was not disclosed to the public. For example, the Hillmen reference (Hillmen et al., N. ENGL. J. MED., 350, pp. 552-59 (2004)) does not disclose any parts of the sequence of eculizumab and the '245 patent (U.S. Pat. No. 6,355,245) does not disclose or even hint at the unique non-naturally occurring, protein-engineered heavy chain (including the CH1-hinge-CH2-CH3 regions) of eculizumab.

4. Nor does the Thomas reference (Thomas et al., MOLECULAR IMMUNOLOGY, 33 (17/18), pp. 1389-1401 (1996)) describe eculizumab. The Thomas reference describes an antibody comprising the humanized variable region of eculizumab, but does not disclose or hint at the unique non-naturally occurring, protein-engineered heavy chain of eculizumab, which confers important functional effects (as discussed below). Rather, the antibody described in the Thomas reference contains a *naturally-occurring* IgG4 heavy chain. The antibody described in the Thomas reference therefore is not eculizumab. It is both structurally and functionally different from eculizumab.

5. As shown below, a sequence alignment of the heavy chain constant regions and hinge of eculizumab (shown in bold on the top line below) and that of the antibody described in the Thomas reference (shown on the bottom line below) demonstrates that the two antibodies are structurally very different, with fourteen different amino acid residues in the constant regions (underlined and highlighted) (four different residues in the CH1 domain, six different residues in the hinge region and four different residues in the CH2 domain):

```
----------------------------CH1-------------------
ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHT
ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHT
```

```
----------CH1-------------------------------|Hinge-
FPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVERKCCV
FPAVLQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKVDKRVESKYGP

-Hinge--|-----------------CH2-----------------
ECPPCPAPP-VAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDP
PCPSCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDP

---------CH2-----------------------------------
EVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKC
EVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKC

--------CH2-------|------------CH3--------------
KVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKG
KVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKG

--------------------------CH3-------------------
FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNV
FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNV

---------CH3-------------
FSCSVMHEALHNHYTQKSLSLSLGK
FSCSVMHEALHNHYTQKSLSLSLGK
```

6. Also, the functional properties of eculizumab and the antibody described in the Thomas reference are very different. For example, eculizumab does not bind as strongly to Fcγ receptors as the Thomas antibody. Binding to the Fcγ receptors initiates and amplifies immune responses that are undesirable for an antibody intended to shut down the terminal complement pathway. Thus, engineering eculizumab to minimize binding to these receptors provided important advantages over the antibody described in the Thomas reference.

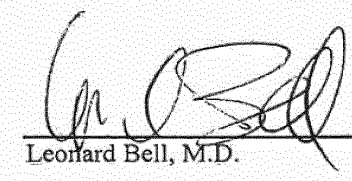
7. Eculizumab also has a much longer plasma half-life than the antibody described in the Thomas reference. It is advantageous for an antibody like eculizumab to remain in the circulation longer so that it does not need to be administered to patients as frequently.

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8. Accordingly, as demonstrated by comparative data between eculizumab and the antibody described in the Thomas reference, the non-natural, protein-engineered, heavy chain of eculizumab reduces effector function activation, minimizes immunogenicity, and contributes to a longer half-life of eculizumab compared to the antibody described in the Thomas reference. These properties were surprising and unpredictable, and made eculizumab a better drug candidate than the antibody described in the Thomas reference.

9. In sum: the complete structure of eculizumab was not available to the public on or before March 15, 2007; eculizumab was not available to the public or being used by the public prior to that time; the unique protein-engineered heavy chain of eculizumab was not described in the Hillmen reference, the Thomas reference or the '245 patent; and the unique protein-engineered heavy chain of eculizumab could not be derived from the combined disclosures of the Hillmen reference, the Thomas reference and/or the '245 patent.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or of any patent issued thereon.



Leonard Bell, M.D. 1/18/2017 Date

D62

DECLARATION

I, Tom Vink, Associate Director, Cell & Molecular Sciences at Genmab B.V. do hereby declare and state:

1. Since 1 July 2002, I am an employee of Genmab B.V., which is an affiliate of Genmab A/S.

2. I have thorough experience within the fields of molecular biology, protein biochemistry and antibody engineering

3. PCT application No. WO 2004/035607 has been disclosed to me.

4. The application relates to human monoclonal antibodies against CD20, and it exemplifies three antibodies, 2F2, 7D8 and 11B8. The application contains claims relating to human monoclonal anti-CD20 antibodies, wherein the antibodies are characterized by the variable heavy chain (V_H) and variable light chain (V_L) amino acid sequences (SEQ ID Nos: 2, 6, 10, 4, 8 and 12), cf. claims 15, 17 and 19 of the application:

	2F2	**7D8**	**11B8**
V_H nucleotide	SEQ ID NO:1	SEQ ID NO:5	SEQ ID NO:9
V_L nucleotide	SEQ ID NO:3	SEQ ID NO:7	SEQ ID NO:11
V_H amino acid	SEQ ID NO:2	SEQ ID NO:6	SEQ ID NO:10
V_L amino acid	SEQ ID NO:4 (identical to SEQ ID NO:8)	SEQ ID NO:8 (identical to SEQ ID NO:4)	SEQ ID NO:12

5. I have been asked whether it would have been obvious for a person skilled in the art at the earliest priority date on 17 October 2002 (i) that the leader sequences erroneously are included in the variable heavy chain and light chain amino acid sequences (SEQ ID Nos: 2, 6, 10, 4, 8 and 12) which define the mature antibodies 2F2, 7D8 and 11B8, respectively, and (ii) in the affirmative, how the variable heavy chain and light chain amino acid sequences without leader sequences should read.

6. For the reasons explained in the following I believe it would have been obvious for a person skilled in the art at the date of the earliest priority date on 17 October 2002 (i) that the leader sequences (marked in red in Appendix A attached hereto) are

included in the variable heavy chain and light chain amino acid sequences, and (ii) how the variable heavy chain and light chain amino acid sequences without leader sequences should read.

7. It is well known that antibodies are secretory proteins, i.e. proteins that are transported to the extracellular medium by passing through the intracellular secretory pathway of the cell, the first compartment of which is the endoplasmic reticulum. Like other secretory proteins, an antibody heavy or light chain protein is initially produced as a precursor polypeptide containing a so called signal or leader peptide (also denoted leader sequence), which is necessary to direct the polypeptide into the endoplasmic reticulum for further transport and secretion. As for other soluble secretory proteins, during the transport into the endoplasmic reticulum, the signal peptides of the antibody heavy and light chains are cleaved off, resulting in a final mature protein (see also page 29, lines 35-36 of the application). In conclusion, any heavy or light chain from an antibody produced by e.g. a hybridoma will be derived from DNA and RNA constructs in which the heavy and light chain encoding sequences are immediately preceded by the leader sequences.

8. To determine the cleavage site between the leader sequence and the antibody sequence so as to determine where the leader sequence stops and where the antibody sequence starts, a comparison can be made with known antibody sequences. Comparison of the protein sequence (precursor polypeptide) containing the leader sequence, as deduced from the cloned RNA sequence, with a database containing known human antibody protein sequences (such as the Vbase as described on page 71, lines 7-8 of the application) will reveal which part of the protein is the signal peptide and where the mature protein starts. At the earliest priority date of the application, several of these databases were available, including the above Vbase as well as the Kabat [1] or IMGT [2] databases.

9. More particularly, these databases contain collections of all germline signal peptides of all human antibodies, and a simple alignment of the derived amino acid sequences (V_H/V_L sequences plus leader sequences) with these signal peptide databases would reveal which part of the sequences are the signal peptides and which part of the sequences are the V_H/V_L sequences. Moreover, comparison/alignment of the derived amino acid sequences (V_H/V_L sequences plus leader sequences) with the collection of mature human V_H and V_L sequences in these databases would reveal where the mature proteins

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start.

As an example, in Figure 1, a selection of V_H germline leader peptides is shown (screenshot from the Vbase). The leader peptide of V_H 7D8, MELGLSWIFLLAILKGVQC, is easily identified as sequence VH3 3-09.

Alternatively, using the DNA plot module on the Vbase site (which, to my knowledge, was available prior to the earliest priority date of 17 October 2002) it is possible to align the nucleotide sequence of your rearranged V gene to its closest mature germline V. In Figure 2 this was done for the V_L region of 11B8 and, as is shown in the screenshot, the start of the mature V_L encoding sequence is gaaatt, confirming the start of the mature V_L polypeptide sequence at the corresponding amino acids EI. This can also be done with the Vquest module at the IMGT site, see Figure 3.

10. The leader sequence for a V_H sequence is typically 19-21 amino acids long, and the leader sequence for a V_L kappa sequence is typically 19-23 amino acids long. A protein always starts with a Met residue, so the leader sequences always start with a Met residue. A V_H sequence may start with a Glu or Gln residue, a V_L kappa sequence may start with Ala, Asp, Val, Asn or Glu. V_H signal peptides can end with Cys, Ala, Ser or Pro, and V_L kappa signal peptides can end with Cys, Ala, Gly or Glu. Accordingly, a person skilled in the art would know from studying the nucleotide and amino acid sequences (SEQ ID Nos: 1-12) that the leader sequences are indeed included. Also a person skilled in the art would note that the amino acid sequences are longer than usual for the mature amino acid sequences. This information was known to a person skilled in the art at the earliest priority date of the application and further supports that the leader sequences are indeed included in the variable heavy chain and light chain amino acid sequences as identified by SEQ ID Nos: 2, 6, 10, 4, 8 and 12, respectively.

11. Alternatively, at the earliest priority date of the application several predictive methods were described, such as [3-5], by which signal peptide sequences could be predicted from a protein sequence. Using the SignalP server (which was available prior to the earliest priority date of 17 October 2002) we have performed this for the V_H polypeptide of antibody 7D8 and a screenshot of the result is shown in Figure 4, indicating the signal peptide cleavage site between the C and E amino acid, confirming the start of the mature V_H region as EV.

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12. In conclusion, it is clear that the claims defining the mature antibodies by the variable heavy chain and light chain amino acid sequences by mistake contain the leader sequences and that nothing else was intended than to define the mature antibodies without these leader sequences.

13. 2F2, 7D8 and 11B8 are characterized by having the following V_H CDR1 regions in the application, cf. for example claims 33, 36 and 40 of the application:

	2F2	**7D8**	**11B8**
V_H CDR1	SEQ ID NO:13	SEQ ID NO:19	SEQ ID NO:25

The CDR regions (SEQ ID Nos: 13-18, 19-24, 25-30) are highlighted in the variable heavy and light chain sequences in Appendix B attached hereto.

14. I have been asked whether it would have been obvious for a person skilled in the art at the earliest priority date on 17 October 2002 (i) that the V_H CDR1 regions erroneously contain an additional amino acid, and (ii) in the affirmative, how the correct V_H CDR1 regions should read.

15. I believe that it would have been obvious for a person skilled in the art at the earliest priority date on 17 October 2002 that (ii) the V_H CDR1 regions contain an additional amino acid, and (ii) how the correct CDR1 regions should read for the below reasons.

16. At the earliest priority date of the application several different methods were available to determine the CDR regions of antibodies. The most common method was the Kabat method [1] (see also [6] page 432-433 for an comprehensive manual for assigning the CDR regions of an antibody using the Kabat numbering scheme). Analysis of SEQ ID Nos. 14, 15, 16, 17, and 18 (2F2), 20, 21, 22, 23, and 24 (7D8) and 26, 27, 28, 29, 30 (11B8) defining the V_H CDR2 and CDR3 regions and the V_L CDR1, CDR2 and CDR3 regions of 2F2, 7D8 and 11B8, respectively, shows that the Kabat numbering scheme has indeed been used in this application.

17. When applying the Kabat numbering rules to the V_H CDR1, cf. [6] page 432: *"Start; Approximately residue 31 (always 9 after a C (Cys))*

Residues before; always CXXXXXXXX (X meaning; any amino acid)
Residue after; Always W. Typically WV, but also WI, WA
Length; 5-7 residues "

It is obvious when applying these rules to the V_H CDR1 regions that the respective V_H CDR1 regions should be "Asp Tyr Ala Met His" for SEQ ID No 13, "Asp Tyr Ala Met His" for SEQ ID No 19 and "Tyr His Ala Met His" for SEQ ID No 25, respectively. When comparing these to the V_H CDR1 sequences as defined in the application, it appears that, by mistake, an extra amino acid has been added to these V_H CDR1 regions.

18. In conclusion, in view of the Kabat rules consistently applied in the application to designate the CDR regions, it is clear that the addition of a further amino acid to the V_H CDR1 sequences was a mistake and that nothing else was intended than to define the V_H CDR1 sequences without inclusion of this additional amino acid.

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

6-Aug-2009

Date

Tom Vink

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References

1. Kabat, E.A., et al., *Sequences of Proteins of Immunological Interest, NIH Publication No. 91-3242.* Bethesda, MD: US Department of Health and Human Services, 1991.
2. Lefranc, M.P., *IMGT, the international ImMunoGeneTics database.* Nucleic Acids Res, 2001. **29**(1): p. 207-9.
3. Chou, K.C., *Prediction of signal peptides using scaled window.* Peptides, 2001. **22**(12): p. 1973-9.
4. Chou, K.C., *Using subsite coupling to predict signal peptides.* Protein Eng, 2001. **14**(2): p. 75-9.
5. Nielsen, H., et al., *Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites.* Protein Eng, 1997. **10**(1): p. 1-6.
6. Kontermann, R. and S. Dübel, *Antibody engineering*. 2001: Springer Heidelberg.

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Apendix A

<210> 1 V_H 2F2
<211> 424
<212> DNA
<213> Homo sapiens

atggagttgg gactgagctg gattttcctt ttggctattt taaaaggtgt ccagtgtgaa 60
gtgcagctgg tggagtctgg gggaggcttg gtacagcctg gcaggtccct gagactctcc 120
tgtgcagcct ctggattcac ctttaatgat tatgccatgc actgggtccg gcaagctcca 180
gggaagggcc tggagtgggt ctcaactatt agttggaata gtggttccat aggctatgcg 240
gactctgtga agggccgatt caccatctcc agagacaacg ccaagaagtc cctgtatctg 300
caaatgaaca gtctgagagc tgaggacacg gccttgtatt actgtgcaaa agatatacag 360
tacggcaact actactacgg tatggacgtc tggggccaag ggaccacggt caccgtctcc 420
tcag 424

<210> 2 V_H 2F2
<211> 141
<212> PRT
<213> Homo sapiens

<400> 2

Met	Glu	Leu	Gly	Leu	Ser	Trp	Ile	Phe	Leu	Leu	Ala	Ile	Leu	Lys	Gly
1				5				10					15		
Val	Gln	Cys	Glu	Val	Gln	Leu	Val	Glu	Ser	Gly	Gly	Gly	Leu	Val	Gln
			20					25					30		
Pro	Gly	Arg	Ser	Leu	Arg	Leu	Ser	Cys	Ala	Ala	Ser	Gly	Phe	Thr	Phe
		35					40					45			
Asn	Asp	Tyr	Ala	Met	His	Trp	Val	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu
	50					55					60				
Glu	Trp	Val	Ser	Thr	Ile	Ser	Trp	Asn	Ser	Gly	Ser	Ile	Gly	Tyr	Ala
65					70					75					80
Asp	Ser	Val	Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ala	Lys	Lys
			85					90					95		
Ser	Leu	Tyr	Leu	Gln	Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp	Thr	Ala	Leu
		100					105					110			
Tyr	Tyr	Cys	Ala	Lys	Asp	Ile	Gln	Tyr	Gly	Asn	Tyr	Tyr	Tyr	Gly	Met
	115					120					125				
Asp	Val	Trp	Gly	Gln	Gly	Thr	Thr	Val	Thr	Val	Ser	Ser			
	130					135					140				

<210> 3 V_L 2F2
<211> 382
<212> DNA
<213> Homo sapiens

<400> 3
atggaagccc cagctcagct tctcttcctc ctgctactct ggctcccaga taccaccgga 60
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ctctcctgca gggccagtca gagtgttagc agctacttag cctggtacca acagaaacct 180
ggccaggctc ccaggctcct catctatgat gcatccaaca gggccactgg catcccagcc 240

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```
aggttcagtg gcagtgggtc tgggacagac ttcactctca ccatcagcag cctagagcct 300
gaagattttg cagtttatta ctgtcagcag cgtagcaact ggccgatcac cttcggccaa 360
gggacacgac tggagattaa ac                                          382
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<210> 4 V_L 2F2
<211> 127
<212> PRT
<213> Homo sapiens

<400> 4

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 1               5                  10                  15
Asp Thr Thr Gly Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser
            20                  25                  30
Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser
        35                  40                  45
Val Ser Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro
    50                  55                  60
Arg Leu Leu Ile Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala
65                  70                  75                  80
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
                85                  90                  95
Ser Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Ser
            100                 105                 110
Asn Trp Pro Ile Thr Phe Gly Gln Gly Thr Arg Leu Glu Ile Lys
        115                 120                 125
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<210> 5 V_H 7D8
<211> 424
<212> DNA
<213> Homo sapiens

<400> 5

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tgtgcagcct ctggattcac ctttcatgat tatgccatgc actgggtccg gcaagctcca 180
gggaagggcc tggagtgggt ctcaactatt agttggaata gtggtaccat aggctatgcg 240
gactctgtga agggccgatt caccatctcc agagacaacg ccaagaactc cctgtatctg 300
caaatgaaca gtctgagagc tgaggacacg gccttgtatt actgtgcaaa agatatacag 360
tacggcaact actactacgg tatggacgtc tggggccaag ggaccacggt caccgtctcc 420
tcag                                                             424
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<210> 6 V_H 7D8
<211> 141
<212> PRT
<213> Homo sapiens

<400> 6

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 1               5                  10                  15
Val Gln Cys Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln
            20                  25                  30
Pro Asp Arg Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
        35                  40                  45
His Asp Tyr Ala Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
    50                  55                  60
Glu Trp Val Ser Thr Ile Ser Trp Asn Ser Gly Thr Ile Gly Tyr Ala
65                  70                  75                  80
Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn
                85                  90                  95
Ser Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Leu
            100                 105                 110
Tyr Tyr Cys Ala Lys Asp Ile Gln Tyr Gly Asn Tyr Tyr Tyr Gly Met
        115                 120                 125
Asp Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
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<210> 7 V_L 7D8
<211> 382
<212> DNA
<213> Homo sapiens

<400> 7

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ctctcctgca gggccagtca gagtgttagc agctacttag cctggtacca acagaaacct 180
ggccaggctc ccaggctcct catctatgat gcatccaaca gggccactgg catcccagcc 240
aggttcagtg gcagtgggtc tgggacagac ttcactctca ccatcagcag cctagagcct 300
gaagattttg cagtttatta ctgtcagcag cgtagcaact ggccgatcac cttcggccaa 360
gggacacgac tggagattaa ac                                          382
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<210> 8 V_L 7D8
<211> 127
<212> PRT
<213> Homo sapiens

<400> 8

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            20                  25                  30
Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser
        35                  40                  45
Val Ser Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro
    50                  55                  60
Arg Leu Leu Ile Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala
65                  70                  75                  80

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
                85                  90                  95
Ser Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Ser
            100                 105                 110
Asn Trp Pro Ile Thr Phe Gly Gln Gly Thr Arg Leu Glu Ile Lys
        115                 120                 125
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<210> 9 V_H 11B8
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<212> DNA
<213> Homo sapiens

<400> 9

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tgtacaggct ctggattcac cttcagttac catgctatgc attgggttcg ccaggctcca 180
ggaaaaggtc tggaatgggt atcaattatt gggactggtg gtgtcacata ctatgcagac 240
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atgaacagcc tgagagccga ggacatggct gtgtattact gtgcaagaga ttactatggt 360
gcggggagtt tttatgacgg cctctacggt atggacgtct ggggccaagg gaccacggtc 420
accgtctcct cag                                                   433
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<210> 10 V_H 11B8
<211> 144
<212> PRT
<213> Homo sapiens

<400> 10

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Val Gln Cys Glu Val Gln Leu Val Gln Ser Gly Gly Gly Leu Val His
            20                  25                  30
Pro Gly Gly Ser Leu Arg Leu Ser Cys Thr Gly Ser Gly Phe Thr Phe
        35                  40                  45
Ser Tyr His Ala Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
    50                  55                  60
Glu Trp Val Ser Ile Ile Gly Thr Gly Gly Val Thr Tyr Tyr Ala Asp
65                  70                  75                  80
Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Val Lys Asn Ser
                85                  90                  95
Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Met Ala Val Tyr
            100                 105                 110
Tyr Cys Ala Arg Asp Tyr Tyr Gly Ala Gly Ser Phe Tyr Asp Gly Leu
        115                 120                 125
Tyr Gly Met Asp Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
    130                 135                 140
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<210> 11 V_L 11B8
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<212> DNA
<213> Homo sapiens

<400> 11

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ctctcctgca gggccagtca gagtgttagc agctacttag cctggtacca acagaaacct 180
ggccaggctc ccaggctcct catctatgat gcatccaaca gggccactgg catcccagcc 240
aggttcagtg gcagtgggtc tgggacagac ttcactctca ccatcagcag cctagagcct 300
gaagattttg cagtttatta ctgtcagcag cgtagcgact ggccgctcac tttcggcgga 360
gggaccaagg tggagatcaa ac                                          382
```


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<210> 12 V_L 11B8
<211> 127
<212> PRT
<213> Homo sapiens

<400> 12

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Asp	Thr	Thr	Gly	Glu	Ile	Val	Leu	Thr	Gln	Ser	Pro	Ala	Thr	Leu	Ser
			20					25					30		
Leu	Ser	Pro	Gly	Glu	Arg	Ala	Thr	Leu	Ser	Cys	Arg	Ala	Ser	Gln	Ser
		35					40					45			
Val	Ser	Ser	Tyr	Leu	Ala	Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Gln	Ala	Pro
	50					55					60				
Arg	Leu	Leu	Ile	Tyr	Asp	Ala	Ser	Asn	Arg	Ala	Thr	Gly	Ile	Pro	Ala
65					70					75					80
Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Asp	Phe	Thr	Leu	Thr	Ile	Ser
				85					90					95	
Ser	Leu	Glu	Pro	Glu	Asp	Phe	Ala	Val	Tyr	Tyr	Cys	Gln	Gln	Arg	Ser
			100					105					110		
Asp	Trp	Pro	Leu	Thr	Phe	Gly	Gly	Gly	Thr	Lys	Val	Glu	Ile	Lys	
		115					120					125			

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Appendix B

<210> 2 V_H 2F2 (Additional amino acid in V_H CDR1 region has been underlined.)
<211> 141
<212> PRT
<213> Homo sapiens

<400> 2

```
Met Glu Leu Gly Leu Ser Trp Ile Phe Leu Leu Ala Ile Leu Lys Gly
 1               5                  10                  15
Val Gln Cys Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln
            20                  25                  30
Pro Gly Arg Ser Leu Arg Leu Ser [Xaa] Ala Ala Ser Gly Phe Thr Phe
        35                  40                  45
Asn Asp Tyr Ala Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
    50                  55                  60
Glu Trp Val Ser Thr Ile Ser Trp Asn Ser Gly Ser Ile Gly Tyr Ala
65                  70                  75                  80
Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Lys
                85                  90                  95
Ser Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Leu
            100                 105                 110
Tyr Tyr Cys Ala Lys Asp Ile Gln Tyr Gly Asn Tyr Tyr Tyr Gly Met
        115                 120                 125
Asp Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
    130                 135                 140
```

<210> 6 V_H 7D8 (Additional amino acid in V_H CDR1 region has been underlined.)
<211> 141
<212> PRT
<213> Homo sapiens

<400> 6

```
Met Glu Leu Gly Leu Ser Trp Ile Phe Leu Leu Ala Ile Leu Lys Gly
 1               5                  10                  15
Val Gln Cys Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln
            20                  25                  30
Pro Asp Arg Ser Leu Arg Leu Ser [Xaa] Ala Ala Ser Gly Phe Thr Phe
        35                  40                  45
His Asp Tyr Ala Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
    50                  55                  60
Glu Trp Val Ser Thr Ile Ser Trp Asn Ser Gly Thr Ile Gly Tyr Ala
65                  70                  75                  80
Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn
                85                  90                  95
Ser Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Leu
            100                 105                 110
Tyr Tyr Cys Ala Lys Asp Ile Gln Tyr Gly Asn Tyr Tyr Tyr Gly Met
        115                 120                 125
Asp Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
    130                 135                 140
```


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<210> 10 V_H 11B8 (Additional amino acid in V_H CDR1 region has been underlined.)
<211> 144
<212> PRT
<213> Homo sapiens

<400> 10

Met	Glu	Leu	Gly	Leu	Ser	Trp	Val	Phe	Leu	Val	Ala	Ile	Leu	Lys	Gly
1				5					10					15	
Val	Gln	Cys	Glu	Val	Gln	Leu	Val	Gln	Ser	Gly	Gly	Gly	Leu	Val	His
			20					25					30		
Pro	Gly	Gly	Ser	Leu	Arg	Leu	Ser	Xxx	Thr	Gly	Ser	Gly	Phe	Thr	Phe
		35					40					45			
Ser	Tyr	His	Ala	Met	His	Trp	Val	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu
	50					55					60				
Glu	Trp	Val	Ser	Ile	Ile	Gly	Thr	Gly	Gly	Val	Thr	Tyr	Tyr	Ala	Asp
65					70					75					80
Ser	Val	Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Val	Lys	Asn	Ser
				85					90					95	
Leu	Tyr	Leu	Gln	Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp	Met	Ala	Val	Tyr
			100					105					110		
Tyr	Cys	Ala	Arg	Asp	Tyr	Tyr	Gly	Ala	Gly	Ser	Phe	Tyr	Asp	Gly	Leu
		115					120					125			
Tyr	Gly	Met	Asp	Val	Trp	Gly	Gln	Gly	Thr	Thr	Val	Thr	Val	Ser	Ser
	130					135					140				

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Figure 1
A screenshot is shown of part of the germline V_H signal peptides in the Vbase database.

VH Leader - Amino acid sequence alignment

		-19 -10 -1
VH1	1-02	MDWTWRILFLVAAATGAHS
	1-03	MDWTWRILFLVAAATGAHS
	1-08	MDWTWRILFLVAAATSAHS
	1-18	MDWTWSILFLVAAPTGAHS
	1-24	MDCTWRILFLVAAATGTHA
	1-45	MDWTWRILFLVAAATDAYS
	1-46	MDWTWRVFCLLAVAPGAHS
	1-58	MDWIWRILFLVGAATGAHS
VH2	2-05	MDTLCSTLLLLTIPSWVLS
	2-26	MDTLCYTLLLLTIPSWVLS
VH3	3-07	MELGLSWVFLVAILEGVQC
	3-09	MELGLSWIFLLAILKGVQC
	3-11	MEFGLSWVFLVAIIKGVQC
	3-13	MELGLSWVFLVAILEGVQC
	3-15	MEFGLSWIFLAAILKGVQC
	3-20	MEFGLSWVFLVAILKGVQC
	3-21	MELGLRWVFLVAILEGVQC
	3-23	MEFGLSWLFLVAILKGVQC
	3-30	MEFGLSWVFLVALLRGVQC
	3-33	MEFGLSWVFLVALLRGVQC

Figure 2
A screenshot of an analysis performed using the DNAplot module on the Vbase site. The complete DNA sequence of the V_L 11B8 antibody was analyzed and an alignment is shown of this sequence with the germline mature V_L regions in the database.

Thank you for using DNAPLOT and V BASE !

Alignment for V segment

EMBL	Locus	Name	score	GAAATTGTGTTGACACAGTCTCCAGCCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGCAGGG
X01668	L6	Vg/38K...+	1305	.....
X17264	L20	Vg"/13K11...+	1278	.....
L19271	-	3A7	1260	.....
L19272	-	3A9	1242	.....G.....
X93639	A27	DPK22/A27...+	1224	.....G.....G.....

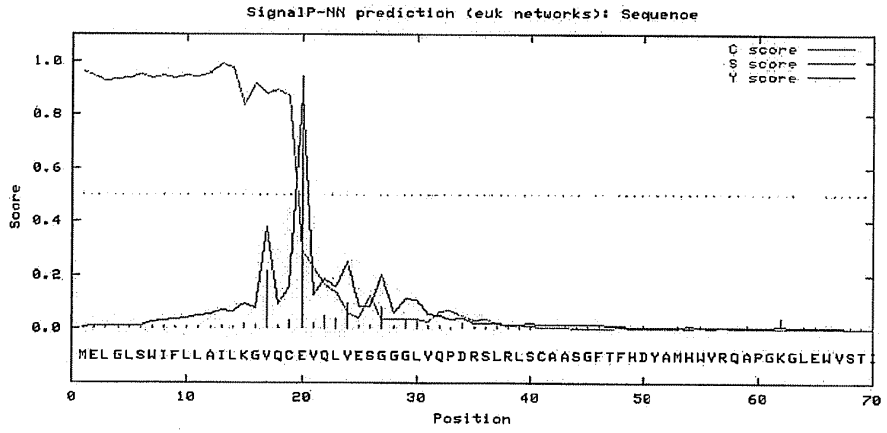
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Figure 3
A screenshot of an analysis performed using the VQUEST module on the IMGT site. The complete DNA sequence of the V_L !1B8 antibody was analyzed and an alignment is shown of this sequence with the germline mature V_L regions in the database.

Using neural networks (NN) and hidden Markov models (HMM) trained on eukaryotes

>Sequence

SignalP-NN result:



data

```
>Sequence                 length = 70
# Measure  Position  Value   Cutoff  signal peptide?
  max. C     20       0.940   0.32    YES
  max. Y     20       0.883   0.33    YES
  max. S     13       0.988   0.87    YES
  mean S      1-19    0.930   0.48    YES
       D      1-19    0.907   0.43    YES
# Most likely cleavage site between pos. 19 and 20: VQC-EV
```

Figure 4
The V_H polypeptide sequence of antibody 7D8 was analyzed using the SignalP server. A screenshot of the resulting prediction of the signal peptide cleavage site is shown.

1. Alignment for V-GENE and allele identification

Closest V-REGIONs (evaluated from the V-REGION first nucleotide to the 2nd-CYS codon plus 15 nt of the CDR3-IMGT)

	Score	Identity
X01668 IGKV3-11*01	1381	99,64% (278/279 nt)
K02768 IGKV3-11*02	1372	99,28% (277/279 nt)
X17264 IGKV3D-11*01	1354	98,57% (275/279 nt)
L19271 IGKV3-NL4*01	1336	97,85% (273/279 nt)
L19272 IGKV3-NL5*01	1318	97,13% (271/279 nt)

Alignment with FR-IMGT and CDR-IMGT delimitations

```
                          <---------------------------------- FR1-IMGT ----------------
VL_11b8                   gaaattgtgttgacacagtctccagccaccctgtctttgtctccaggggaaagagccacc
X01668 IGKV3-11*01        ---------------------------------------------------------
K02768 IGKV3-11*02        ---------------------------------------------------------
X17264 IGKV3D-11*01       ---------------------------------------------------------
L19271 IGKV3-NL4*01       ---------------------------------------------------------
L19272 IGKV3-NL5*01       ----------g----------------------------------------------

                          ---------->_____________ CDR1-IMGT _________<-----
VL_11b8                   ctctcctgcagggccagtcagagtgtt.................agcagctacttagcc
X01668 IGKV3-11*01        -------------------------.................----------------
K02768 IGKV3-11*02        -------------------------.................----------------
X17264 IGKV3D-11*01       ----------------------g----.................----------------
```


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Drugs

Denosumab

Targets (1)

IDENTIFICATION

Name	Denosumab
Accession Number	DB06643
Type	Biotech
Groups	Approved
Biologic Classification	Protein Based Therapies **Monoclonal antibody (mAb)**
Description	Denosumab is a novel, fully human IgG2 monoclonal antibody specific to receptor activator of nuclear factor kappa-B ligand (RANKL), suppresses bone resorption markers in patients with a variety of metastatic tumors and is being investigated in multiple clinical trials for the prevention and treatment of bone metastases. Chemically, it consists of 2 heavy and 2 light chains. Each light chain consists of 215 amino acids. Each heavy chain consists of 448 amino acids with 4 intramolecular disulfides. FDA approved on June 1, 2010.
Protein structure	
Protein chemical formula	$C_{6404}H_{9912}N_{1724}O_{2004}S_{50}$
Protein average weight	144700.0 Da
Sequences	> Denosumab αOPGL-1 heavy chain sequence EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSGITGSGGSTYY ADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKDPGTTVIMSWFDPWGQGTLVTV SSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQ SSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELL GGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR DELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKS RWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK > Denosumab αOPGL-1 light chain sequence EIVLTQSPGTLSLSPGERATLSCRASQSVRGRYLAWYQQKPGQAPRLLIYGASSRATGIP DRFSGSGSGTDFTLTISRLEPEDFAVFYCQQYGSSPRTFGQGTKVEIKRTVAAPSVFIFP PSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTL TLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC Download FASTA Format
Synonyms	Not Available
External IDs	AMG-162

Prescription Products

Show 10 entries Search

NAME	DOSAGE	STRENGTH	ROUTE	LABELLER	MARKETING START	MARKETING END
Prolia	Solution	60 mg	Subcutaneous	Amgen	2010-08-12	Not applicable

Indication

Prolia is indicated for the treatment of postmenopausal women with osteoporosis at high risk for fracture. It reduces the incidence of vertebral, nonvertebral, and hip fractures. Prolia is also indicated as a treatment to increase bone mass in women at high risk for fracture receiving adjuvant aromatase inhibitor therapy for breast cancer. It can also be used in men with osteoporosis at high risk for fracture or in men receiving androgen deprivation therapy for nonmetastatic prostate cancer to increase bone mass. Xgeva is indicated for the prevention of skeletal-related events in patients with bone metastases from solid tumors.

Associated Conditions

Bone Loss

Bone pain

Fracture Bone

Spinal Cord Compression

Bone destruction

Giant cell tumor of the bone

High risk of fracture Osteoporosis

Refractory Hypercalcemia of malignancy

Pharmacodynamics

In clinical studies, treatment with 60 mg of Prolia resulted in reduction in the bone resorption marker serum type 1 C-telopeptide (CTX) by approximately 85% by 3 days. Consistent with the physiological coupling of bone formation and resorption in skeletal remodeling, subsequent reductions in bone formation markers (i.e. osteocalcin and procollagen type 1 N-terminal peptide [PINP]) were observed starting 1 month after the first dose of Prolia.

Mechanism of action

Denosumab is designed to target RANKL (RANK ligand), a protein that acts as the primary signal to promote bone removal/resorption. In many bone loss conditions, RANKL overwhelms the body's natural defense against bone destruction. Denosumab prevents RANKL from activating its receptor, RANK, on the surface of osteoclasts and their precursors. Prevention of the RANKL/RANK interaction inhibits osteoclast formation, function, and survival, thereby decreasing bone resorption and increasing bone mass and strength in both cortical and trabecular bone.

TARGET	ACTIONS	ORGANISM
(A) Tumor necrosis factor ligand superfamily member 11	antibody	Humans

ADDITIONAL DATA AVAILABLE

Adverse Effects

Comprehensive structured data on known drug adverse effects with statistical prevalence. MedDRA and ICD10 ids are provided for adverse effect conditions and symptoms.

LEARN MORE

ADDITIONAL DATA AVAILABLE

Contraindications

Structured data covering drug contraindications. Each contraindication describes a scenario in which the drug is not to be used. Includes restrictions on co-administration, contraindicated populations, and more.

LEARN MORE

ADDITIONAL DATA AVAILABLE

Blackbox Warnings

Structured data representing warnings from the black box section of drug labels. These warnings cover important and dangerous risks, contraindications, or adverse effects.

LEARN MORE

Absorption

When 60 mg of denosumab was subcutaneously administered to healthy subjects after fasting for 12 hours, the pharmacokinetic parameters are as follows: Cmax = 6.75 mcg/mL; Tmax= 10 days (range of 3 to 21 days); AUC (0-16 weeks) = 316 mcg•day/mL. Denosumab does not accumulate following multiple doses once every 6 months. The pharmacokinetics of denosumab were not affected by the formation of antibodies.

Volume of distribution

Not Available

Protein binding

Not Available

Metabolism

Not Available

Route of elimination

Not Available

INTERACTIONS

Drug Interactions

Drugs

This information should not be interpreted without the help of a healthcare provider. If you believe you are experiencing an interaction, contact a healthcare provider immediately. The absence of an interaction does not necessarily mean no interactions exist.

ALL DRUGS | APPROVED | VET APPROVED | NUTRACEUTICAL | ILLICIT | WITHDRAWN | INVESTIGATIONAL | EXPERIMENTAL

Show 10 entries

Search

DRUG	INTERACTION
2-Methoxyethanol	The risk or severity of adverse effects can be increased when Denosumab is combined with 2-Methoxyethanol.
9-(N-methyl-L-isoleucine)-cyclosporin A	The risk or severity of adverse effects can be increased when Denosumab is combined with 9-(N-methyl-L-isoleucine)-cyclosporin A.
Abatacept	The risk or severity of adverse effects can be increased when Denosumab is combined with Abatacept.
Abciximab	The risk or severity of adverse effects can be increased when Abciximab is combined with Denosumab.
Abetimus	The risk or severity of adverse effects can be increased when Denosumab is combined with Abetimus.
Abituzumab	The risk or severity of adverse effects can be increased when Denosumab is combined with Abituzumab.
Abrilumab	The risk or severity of adverse effects can be increased when Denosumab is combined with Abrilumab.
Acteoside	The risk or severity of adverse effects can be increased when Denosumab is combined with Acteoside.
Adalimumab	The risk or severity of adverse effects can be increased when Denosumab is combined with Adalimumab.
Adecatumumab	The risk or severity of adverse effects can be increased when Adecatumumab is combined with Denosumab.

ADDITIONAL DATA AVAILABLE

Showing 1 to 10 of 590 entries

< 1 2 3 4 5 ... 59 >

Food Interactions Not Available

REFERENCES

General References

1. Malan J, Ettinger K, Naumann E, Beirne OR: The relationship of denosumab pharmacology and osteonecrosis of the jaws. Oral Surg Oral Med Oral Pathol Oral Radiol. 2012 Dec;114(6):671-6. doi: 10.1016/j.oooo.2012.08.439. [PubMed:23159111]
2. Link [Link]
3. Link [Link]

External Links

KEGG Drug	D03684
PubChem Substance	347910354
ChEMBL	CHEMBL1237023
PharmGKB	PA166048634
RxList	RxList Drug Page
Drugs.com	Drugs.com Drug Page
Wikipedia	Denosumab

ATC Codes

M05BX04 — Denosumab

- M05BX — Other drugs affecting bone structure and mineralization
- M05B — DRUGS AFFECTING BONE STRUCTURE AND MINERALIZATION
- M05 — DRUGS FOR TREATMENT OF BONE DISEASES
- M — MUSCULO-SKELETAL SYSTEM

CLINICAL TRIALS

Drugs

Clinical Trials Show 10 entries Search

PHASE	STATUS	PURPOSE	CONDITIONS	COUNT
0	Completed	Basic Science	Aging / Bone Loss	1
0	Recruiting	Prevention	BRCA1 Gene Mutation / Brca2 Gene Mutation / Fallopian Tube Carcinoma / Ovarian Carcinoma / Premenopausal	1
0	Recruiting	Treatment	Anorexia Nervosa (AN) / Atypical Anorexia Nervosa / Bone Density / Bone Loss / Eating Disorder	1
0	Recruiting	Treatment	Breast Neoplasm Female / Hormone Receptor Negative Neoplasm / Hormone Receptor Positive Tumor / Stage I Breast Carcinoma / Stage II Breast Cancer	1
0	Recruiting	Treatment	Cancer, Breast	1
1	Active Not Recruiting	Treatment	Advanced Cancers / Advanced Malignant Neoplasm / Metastatic Malignant Neoplasm / Recurrent Malignant Neoplasm / Refractory Malignant Neoplasm	1
1	Active Not Recruiting	Treatment	Postmenopausal Osteoporosis (PMO)	1
1	Completed	Not Available	Healthy Volunteers	1
1	Completed	Basic Science	Healthy Volunteer, Female, Breast	1
1	Completed	Basic Science	Hypersensitivity, Delayed / Immune Tolerance/Drug Effects / Immunosuppression / Ultraviolet Rays	1

Showing 1 to 10 of 149 entries < 1 2 3 4 5 ... 15 >

PHARMACOECONOMICS

Manufacturers Not Available

Packagers Not Available

Dosage forms Show 10 entries Search

FORM	ROUTE	STRENGTH
Injection	Subcutaneous	60 mg/1mL
Solution	Subcutaneous	60 mg
Injection	Subcutaneous	120 mg/1.7mL
Injection, solution	Subcutaneous	120 mg
Solution	Subcutaneous	120 mg

Showing 1 to 5 of 5 entries < 1 >

Prices Not Available

Patents Show 10 entries Search

PATENT NUMBER	PEDIATRIC EXTENSION	APPROVED	EXPIRES (ESTIMATED)
CA2257247	No	2012-09-11	2018-04-15
CA2274987	No	2012-01-24	2017-12-22
CA2265274	No	2010-09-28	2018-04-15
CA2400920	No	2011-05-31	2021-02-23
CA2328140	No	2012-03-13	2019-05-13

Showing 1 to 5 of 5 entries < 1 >

PROPERTIES

State	Solid
Experimental Properties	Not Available

Drugs

TAXONOMY

Description	Not Available
Kingdom	Organic Compounds
Super Class	Organic Acids
Class	Carboxylic Acids and Derivatives
Sub Class	Amino Acids, Peptides, and Analogues
Direct Parent	Peptides
Alternative Parents	Not Available
Substituents	Not Available
Molecular Framework	Not Available
External Descriptors	Not Available

TARGETS

1. Tumor necrosis factor ligand superfamily member 11

Details

Kind	Protein	**General Function**	Tumor necrosis factor receptor superfamily binding
Organism	Humans	**Specific Function**	Cytokine that binds to TNFRSF11B/OPG and to TNFRSF11A/RANK. Osteoclast differentiation and activation factor. Augments the ability of dendritic cells to stimulate naive T-cell proliferation. May be...
Pharmacological action	Yes	**Gene Name**	TNFSF11
Actions	Antibody	**Uniprot ID**	O14788
		Uniprot Name	Tumor necrosis factor ligand superfamily member 11
		Molecular Weight	35477.81 Da

References

1. Lipton A, Jun S: RANKL inhibition in the treatment of bone metastases. Curr Opin Support Palliat Care. 2008 Sep;2(3):197-203. doi: 10.1097/SPC.0b013e32830baac2. [PubMed 18685421]
2. Westenfeld R, Ketteler M, Brandenburg VM: Anti-RANKL therapy--implications for the bone-vascular-axis in CKD? Denosumab in post-menopausal women with low bone mineral density. Nephrol Dial Transplant. 2006 Aug;21(8):2075-7. Epub 2006 May 15. [PubMed 16702192]

Drug created on March 13, 2005 10:43 / Updated on November 03, 2019 20:56

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This project is supported by the Canadian Institutes of Health Research (award #111062), Alberta Innovates - Health Solutions, and by The Metabolomics Innovation Centre (TMIC), a nationally-funded research and core facility that supports a wide range of cutting-edge metabolomic studies. TMIC is funded by Genome Alberta, Genome British Columbia, and Genome Canada, a not-for-profit organization that is leading Canada's national genomics strategy with funding from the federal government. Maintenance, support, and commercial licensing is provided by OMx Personal Health Analytics, Inc. Designed by Educe Design & Innovation Inc.

BESCHWERDEKAMMERN DES EUROPÄISCHEN PATENTAMTS

BOARDS OF APPEAL OF THE EUROPEAN PATENT OFFICE

CHAMBRES DE RECOURS DE L'OFFICE EUROPEEN DES BREVETS

A		B	X	C	

D61

File Number: T 386/89 - 3.2.1

Application No.: 80 303 122.8

Publication No.: 0 025 677

Title of invention: Wheels manually adjustable for varying track

Classification: B60B 23/12, B60B 35/10

D E C I S I O N
of 24 March 1992

Proprietor of the patent: GKN Sankey Limited

Opponent: 01 Lemmerz-Werke KGaA
02 Levypyörä OY

Headword: Tractor wheel/SANKEY

EPC Article 56

Keyword: "Complete reformulation of problem in terms of an alleged effect not deducible from original disclosure (not allowed)"

Catchword

The alleged effect of a described feature cannot be taken into account when determining the problem underlying the invention for the purpose of assessing inventive step, if it cannot be deduced by the skilled person from the application as filed considered in relation to the nearest prior art.

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Case Number : T 386/89 - 3.2.1

D E C I S I O N
of the Technical Board of Appeal 3.2.1
of 24 March 1992

Appellant : (Proprietor of the patent)	GKN Sankey Limited P.O. Box 20 Hadley Castle Works Telford, Shropshire TF1 4RE (GB)
Representative :	Thorpe, Brian Forrester & Boehmert Widenmayerstraße 4 W-8000 München 22 (DE)
Respondent : (Opponent 01)	Lemmerz-Werke KGaA Ladestraße 1 W-5330 Königswinter 1 (DE)
Representative :	Buschhoff, Josef, Dipl.-Ing. Patentanwälte Dipl.-Ing. Buschhoff Dipl.-Ing. Hennicke, Dipl.-Ing. Vollbach Kaiser-Wilhelm-Ring 24 Postfach 190 408 W-5000 Köln 1 (DE)
Respondent : (Opponent 02)	Levypyörä OY PL 44 FI-15561 Nastola (FI)
Representative :	Grams, Klaus Dieter, Dipl.-Ing. Patentanwaltsbüro Tiedtke-Bühling-Kinne-Grupe-Pellmann-Grams-Struif-Winter-Roth Bavariaring 4 W-8000 München 2 (DE)
Decision under appeal :	Decision of Opposition Division of the European Patent Office dated 25 January 1989, with written reasons posted on 27 April 1989 revoking European patent No. 0 025 677 pursuant to Article 102(1) EPC.

Composition of the Board :

Chairman : F. Gumbel
Members : M. Ceyte
W. Moser
S. Crane
J.C. De Preter

Summary of Facts and Submissions

I. European patent No. 0 025 677 was granted on 2 January 1985 on the basis of European patent application No. 80 303 122.8.

II. The patent was opposed by the Respondents 01 and 02 on the ground that its subject-matter did not involve an inventive step. In support of their requests, they referred inter alia to the following prior art:

- D1, "Fertigungszeichnung No. 247 938 713" of Rheinstahl Hanomag AG, 15 July 1966 (D1 was filed as "Anlage 14" by Opponent I with a letter dated 22 May 1985) and

- the wheel shown in Figure 1 of the patent specification, which represents prior art.

III. The patent was revoked by a decision taken at the oral proceedings on 25 January 1989 with written reasons posted on 27 April 1989.

IV. The Appellant (proprietor of the patent) filed an appeal against this decision on 14 June 1989 and paid the appeal fee on the same day. The Statement of Grounds of Appeal was filed on 25 August 1989.

V. In a communication pursuant to Article 11(2) of the Rules of Procedure dispatched on 7 November 1991, the Board, applying the principle of examination by the European Patent Office of its own motion as set out in Article 114(1) EPC, introduced into the proceedings the further document:

- DE-C-928 868 (D3)

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which was cited in the search report, taken into consideration during the examination proceedings and discussed in the specification of the patent in suit.

VI. At the oral proceedings held on 24 March 1992 the Appellant requested that the decision under appeal be set aside and that the patent be maintained on the basis of conditional Claim I filed with the Statement of Grounds.

A request for reimbursement of the appeal fee was withdrawn.

The Respondents requested that the appeal be dismissed.

VII. The Appellant's arguments set forth in his written and oral statements can be summarised as follows:

The problem with which the invention is concerned is the increase of the fatigue life of a manually adjustable wheel for a vehicle, in particular for a high powered tractor, by which the track of the vehicle can be adjusted.

A wheel with inadequate fatigue life will suffer failure sooner than is acceptable but the type of failure is also of significance. A failure of one of the bolts securing the disc to the lugs might be inconvenient but occurs infrequently and in any case is not serious since a replacement can readily be obtained and is easily fitted. On the other hand, a failure of the rim, the disc or the lugs would normally require the purchase of a new wheel.

In practice, it was found that failures most commonly occurred at the points of attachment of the lugs to the rim. Either the lugs became detached or the rim cracked at

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the point of lug attachment (see Mr E. Williams' Affidavit filed during the opposition proceedings). Hence, at the time when the invention was made, a skilled person attempting to improve the fatigue life of the wheels of high-powered tractors would most likely have turned his attention to the lugs and their attachment to the rim. In particular, the skilled person would have tried to minimise the maximum stress range in each lug and its attachment as the wheel rotates under load and to maximise the ability of the lug and its attachment to withstand this stress range. In doing this, the skilled person would, of course, be constrained by cost considerations since a customer would not accept significant cost increases even if a somewhat improved fatigue life could be achieved. In order to achieve fatigue life improvements, the skilled person might have considered increasing the number of lugs thus reducing the stress peaks by distributing the total load over more lugs but would then have realised that this would require not only more lugs, but also more welding of lugs to the rim, more bolts and possibly more disc material so that the costs would increase considerably. It might also be considered that bigger lugs could be used while retaining the same number of lugs but clearly this would increase material and welding costs. The same applies for an increase of thickness of material for the rim and the lugs.

That the invention actually solves the problem of increasing the fatigue life of the wheels of high-powered tractors without an unacceptable additional cost is clear from the comparative fatigue life tests made and the fact that nevertheless costs were reduced (see the Williams Affidavit - paragraphs 8, 13 and Exhibits A and C).

There is no disclosure or suggestion of a prior art wheel that does not have its lugs equally spaced around the

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rim. Thus, if four lugs are used, as is the case with the wheel according to D1, they are at 90 degree angles, six lugs at 60 degree angles and so on. Tractor wheel designers had considered that the lugs must be equally spaced since any other arrangement would apparently cause uneven loading of the lugs with resultant stress peaks in certain areas which would lead to a decrease in the expected fatigue life.

The irregular octagonal disc of the D1 wheel, while it may save disc material in wheels with 4 lugs, was not seen as having any fatigue life increasing properties.

If the skilled person were to consider the possibility of modifying the D1 wheel to provide a wheel with higher fatigue life for high powered tractors, he would have had the possibilities of thickening the rim, altering the disc, increasing the number of lugs, increasing the size of the lugs, or altering the welding of the lugs to the rim. None of these possibilities lead towards the invention.

The impugned Decision suggests that the skilled person starting from D1 might have considered adopting the type of lug used in the wheel of Figure 1 of the patent without giving any reason why he should adopt just this type of lug, even though many other possibilities exist. This is however unlikely and the provision of twice as many lugs would be resisted on cost grounds. Furthermore, the provision of the lugs in pairs would be resisted on the ground that it would be expected to reduce the fatigue life (see discussion in the previous sections).

The wheel of Figure 1 of the patent in suit also has equi-angularly spaced lugs. Thus this prior art document similarly contains no suggestion that the above defined

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technical problem can be solved by a wheel having its lugs unequally spaced in pairs about the rim.

VIII. The Respondents contested the Appellant's arguments and particularly took the view that the idea of using separate lugs in the case of bolts arranged in pairs as known from D1 did not imply an inventive step, considering the prior art according to Figure 1 of the patent in suit.

Although in the Williams Affidavit filed by the Appellant during the opposition proceedings it is specified that wheels which do not have lugs equally spaced around the rim have an increased fatigue life if compared with wheels having equi-angularly spaced lugs, the comparative tests referred to in this affidavit did not provide a fair basis for this conclusion. In any case, the finding of an additional useful and unexpected effect was not necessarily indicative of inventiveness (see T 21/81, OJ EPO 1983, 15) if a claimed solution was suggested by the prior art for other reasons.

In the present case the teachings of the state of the art according to D1 and Figure 1 of the patent led directly to the adoption of lugs arranged in pairs to solve the technical problem actually underlying the patent in suit, i.e. the reduction of material consumption.

Moreover this additional effect was nowhere mentioned in the documents as filed and could also not be deduced by a skilled person from the original application as filed. Hence, it could not be taken into account when assessing the issue of inventive step.

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IX. Valid Claim 1 reads as follows:

"1. A manually adjustable wheel for a vehicle, e.g. a tractor, the wheel comprising a rim (51) to receive a tyre, fixing lugs (58) spaced apart around the radially inner periphery of the rim, the lugs being of equal length considered parallel to the rotary axis of the wheel with their one ends co-planar and their other ends co-planar, each lug being welded directly to the rim, a disc (50) of uniform thickness for connection to a hub of the vehicle, the disc having a generally flat outer portion (54) which is generally perpendicular to the rotary axis of the wheel, and bolt means (61, 65) detachably securing the disc to the lugs, the disc and the lugs being arranged so that the disc may be selectively secured to the lugs in either of two axially spaced positions, each lug being channel-shaped and having a flange formed integrally with each wall of the channel, the flanges overlying the inner surface of the rim and being welded thereto, and each lug receiving a single bolt of the bolt means, characterised in that: the shape of the disc is that of a square with the corners cut off so that the disc has an irregular octagonal shape which has four longer sides (56) and four shorter sides (57) arranged alternately around the periphery of the octagon, and the lugs are arranged in four pairs so that the circumferential spacing between the lugs in a pair is substantially less than the circumferential spacing between adjacent lugs of adjacent pairs, each of the shorter sides of the disc being juxtaposed against the ends of a pair of lugs, and the two bolts received by that pair of lugs passing through one said shorter side of the disc."

X. At the conclusion of the oral proceedings the Board's decision to dismiss the appeal was announced.

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Reasons for the Decision

1. The appeal complies with Articles 106 to 108 and Rule 64 EPC and is admissible.

2. Article 123

During the oral proceedings, the Respondents raised the objection that the amendments to the characterising portion of Claim 1 concerning the addition of the term "substantially" and the excision of the requirement that the bolts are "only" located along the shorter sides of the disc did not meet the provisions of Article 123.

However, the Board is of the opinion that there is sufficient support for the term "substantially" in the application as originally filed (see original Figures 6 and 8) and that the extent of protection conferred is not extended by the omission of the word "only" since it is clear from the patent specification and the claims as amended that the bolts cannot be situated along the longer sides of the disc because there are no lugs here.

3. Novelty

Neither the wheel shown in Figure 1 of the patent in suit nor the D1 or D3 wheels disclose a manually adjustable wheel having all the features specified in Claim 1. Therefore the subject-matter of Claim 1 is novel within the meaning of Article 54 EPC. This was, in fact, not put into question by the Respondents during the opposition and appeal proceedings.

4. Problem and Solution

4.1 Contrary to the teachings of the original disclosure in which the sole emphasis was on the achievement of material

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savings with respect to the disc the Appellant now argues that the technical problem solved by the invention is to be seen in the enhancement of the fatigue life of the wheel through the arrangement of the lugs in pairs, this being irrespective of whether the wheel of D1 or of Figure 1 of the patent in suit is taken as the closest state of the art.

4.2 There can be no doubt that the feature that the lugs are arranged in pairs is, as such, disclosed in the patent application as originally filed. Such a feature is clearly shown or described in particular in the embodiments of original Figures 6 and 8. At the oral proceedings the Appellant contended that the effect of enhancement of fatigue life can be derived from the statement at page 1 of the application as originally filed according to which the manually adjustable wheel is "suitable for use on tractors of high power". In the Board's opinion, the technical significance concerning the increase of fatigue life through the arrangement in pairs of the bolts is not derivable from such a statement by a skilled person. This effect is also not self-evident: the Appellant himself has admitted that the increase in fatigue life was surprising and is indeed still not fully understood. Furthermore, no distinction is made in this context between the embodiments of original Figures 6 and 8 and that of original Figure 3 where a single lug rather than a pair of lugs is associated with each short side of the disc.

There is no disclosure or suggestion in the application as originally filed that this feature or indeed any other feature was in any way associated with increasing the fatigue life of the wheel.

4.3 It belongs to the well-established jurisprudence of the Boards of Appeal that where a specific problem is identified in the description, the applicant or patentee

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may be allowed to put forward a modified version of the problem particularly if the issue of inventiveness has to be considered on an objective basis against a new prior art which comes closer to the invention than that considered in the original patent application or granted patent specification. Reference is made in this respect to the decision T 184/82 (OJ EPO 1984, 261) where the Board allowed a re-definition of the problem to such an extent that the skilled person "could recognise the same as implied or related to the problem initially suggested" (see point 5 of the reasons).

In the decision T 13/84 (OJ EPO 1986, 253) the Board held that the reformulation of the problem is not precluded by Article 123(2) EPC if the problem could be deduced by a person skilled in the art from the application as originally filed when seen in the light of the nearest prior art (see point 11). The same was confirmed in the decisions T 547/90 of 17 January 1991 (unpublished) and T 469/90 of 6 February 1991 (unpublished).

According to the above decisions, the reformulation of the problem would not contravene Article 123(2), that is to say would not constitute added subject-matter when amending the statement of the problem in the description as long as the problem can be clearly deduced by the skilled person from the application as originally filed when considered in relation to the nearest prior art.

Applying the above principles, the Board in the present case concludes that the alleged unexpected effect, i.e. the improved fatigue life, which as indicated above is not deducible from the application as originally filed, cannot be taken into account when determining the problem underlying the invention for the purpose of assessing the issue of inventive step.

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Hence, the problem actually to be taken into account is in essence still the same as that expressly mentioned in the application as originally filed in relation to the Figure 1 wheel which is regarded as the nearest prior art by the Appellant. According to this problem the amount of material used for the disc shall be optimised having regard to the required strength of the wheel, in particular when used on tractors of high power.

4.4 According to the Appellant's submissions valid Claim 1 comprises in essence the following features:

1. a rim,
2. fixing lugs welded to the rim,
3. a disc,
4. bolts securing the disc to the lugs,
5. the disc has an irregular octagonal shape,
6. the lugs are channel shaped,
7. the lugs have flanges welded to the rim,
8. there are 8 lugs,
9. the lugs are arranged in pairs.

The Figure 1 wheel, against which the preamble of Claim 1 is correctly delimited, comprises a wheel rim provided on its radially inner surface with a set of eight equi-distantly spaced apart lugs welded to the rim. The disc which is detachably secured to the rim lugs by means of bolts, is of generally circular configuration but has a scalloped periphery with the apices of the scallops providing portions adjacent the rim which are boltable to the lugs (column 2, lines 42-50 of the patent specification).

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According to the description as originally filed (page 4, lines 1-4) and the patent specification (column 2, lines 61-65), "the construction of the disc 14 is extremely wasteful of metal since it is generally circular and is usually produced as a steel stamping out of a square of rectangular blank". The Appellant has set out to remove this disadvantage (see statement of problem above).

4.5 According to the characterising portion of Claim 1 this problem is in essence solved by the above features (5) and (9), that is to say:

5. the disc has an irregular octagonal shape,
9. the lugs are arranged in pairs.

5. Inventive step

5.1 The provision of an "octagonal" disc (above feature (5)) was already known from the undisputed prior use of a wheel according to D1 and from document D3. Referring in particular to Figure 3 of document D3, it will be seen that the shape of the disc is that of an irregular octagon having four long sides and four short sides constituted by the corner portions, the long sides and short sides arranged alternately around the periphery of the disc.

It is expressis verbis stated in this document that a disc of this shape provides the advantage of saving material (column 1, lines 8, 9).

Thus the "octagonal" design (feature (5)) proposed in this citation is immediately suggested to the skilled person trying to solve the problem underlying the disputed patent.

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5.2 Having regard now to the remaining feature (9), i.e. the arrangement in pairs of the lugs, any skilled person would be aware that once having chosen the octagonal shape of the disc the eight lugs of the Figure 1 wheel can only be located on the four shorter sides of the disc, because only these shorter sides come into contact with the rim, and that each shorter side is to be provided with a pair of lugs (since 8 lugs are to be distributed on the 4 shorter sides). Thus the idea of arranging the lugs (or the corresponding bolts) in pairs is imposed by the design of the disc. Therefore the Board considers that feature (9) merely represents a mandatory step not apt to impart inventiveness to the claimed subject-matter.

5.3 The Appellant argues that it is questionable whether the skilled person would in fact have considered retaining the same number and type of lugs and producing an uneven spacing since an uneven spacing of the lugs would have been thought unacceptable according to the conventional wisdom that only even lug spacing would equalize the stress across each lug and thereby reduce the stress peaks occurring at the welds between the rim and the lugs. This argument is not convincing to the Board: The claimed number of bolts (8) and the claimed number of lugs (8) are known from the nearest prior art, i.e. the Figure 1 wheel used as starting point. There would be no sound reason to deviate from the teaching of this nearest prior art and thus to provide a modified number of bolts and lugs. Moreover as to the arrangement of the bolts with a disc having an irregular octagonal shape, the wheel according to D1 also has them arranged in pairs at the shorter sides of the disc. The fact that in D1 unitary "boat lugs" are used for each pair of bolts instead of separate channel-shaped lugs as used with the invention and as known from the Figure 1 wheel does not detract from that general teaching.

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5.4 No general prejudice that might have prevented a wheel designer from pairing the lugs can be seen by the Board and the Appellant did not bring forward any evidence in support of such a prejudice.

5.5 As pointed out above under point 4.3, in the present case the effect concerning the improved fatigue life cannot be taken into account for reformulation of the problem when assessing inventive step. Hence, the argument that the skilled person would have considered different solutions rather than arranging the lugs in pairs when trying to enhance fatigue life of the wheel, need not be dealt with.

5.5.1 However, even if this effect were to be taken into consideration as an additional surprising effect, the Board considers that it merely represents a "bonus effect" which would not be apt to impart inventiveness to the claimed subject-matter. Reference is made in this context to the decision T 21/81 (OJ EPO 1983, 15) where it is stated (point 6 of the reasons) that

"if having regard to the state of the art, it would already have been obvious for a skilled person in the art to arrive at something falling within the terms of a claim, because an advantageous effect (here, the reduction of material consumption) could be expected from the combination of the teachings of the prior art documents (here, Figure 1 wheel and document D3 or D1), such claim lacks inventive step irrespective of the circumstance that an extra effect (possibly unforeseen) is obtained".

Hence, since in the present case the advantageous effect of a reduction of material consumption while maintaining good mechanical properties could be expected by combining

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teachings of the prior art, the extra effect of an improved service life cannot render the subject-matter of present Claim 1 inventive.

5.6 In these circumstances there is no necessity to investigate whether the alleged effect is properly demonstrated as being related to the features claimed by the comparative test according to Table C annexed to Mr Williams' affidavit and discussed therein.

5.7 For the above reasons the Board comes to the conclusion that the subject-matter of Claim 1 does not involve an inventive step within the meaning of Article 56 EPC. As the Board is bound by the single request of the Appellant it is not necessary to consider the merits of the subject-matter of dependent Claims 2 and 3.

6. The Board is thus of the opinion that the grounds for opposition prejudice the maintenance of the patent.

Order

For these reasons, it is decided that:

The appeal is dismissed.

The Registrar: The Chairman:

S. Fabiani F. Gumbel

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Munich, November 08, 2019

Our Ref.:	**198 441 u6/m6** **Eculizumab Composition / ALEXION**
Opposition to:	**EP 2 359 834 B1 (11001632.6)**
Appeal Case:	**T 1087/19-3.3.01**
Proprietor:	**Alexion Pharmaceuticals, Inc.**
Opponent 2:	**Hoffmann Eitle** **Patent- und Rechtsanwälte PartmbB** **Arabellastr. 30** **81925 Munich (DE)**

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In Response to the Grounds of Appeal filed by the Patent Proprietor on June 24, 2019

1 REQUESTS

(1) The present Respondent agrees with the Decision of the first instance insofar as it is adverse to the Appellant. The Respondent requests dismissal of the appeal. Oral proceedings are requested in the event that the Board considers anything other than dismissal of the Appeal. It is also requested to not admit auxiliary requests 1 - 5, as well as Auxiliary Request 7 into the proceedings; all these auxiliary requests are late filed. It is furthermore requested to not admit documents **D33** as well as **D54 – 60** into the proceedings, as they are late filed.

2 REASONS

2.1 Late Filed Requests and Documents

2.1.1 Late Filed Requests

(2) The Proprietor has submitted a Main Request and 8 Auxiliary Requests with the Grounds of Appeal; of these requests, only the Main Request and Auxiliary Requests 6 and 8 were admitted into the proceedings during first instance. Auxiliary Request 6 corresponds to Auxiliary Request 1 before the Opposition Division and Auxiliary Request 8 corresponds to Auxiliary Request 2 before the Opposition Division. No further requests were pending in first instance proceedings.

(3) Auxiliary Request 1 now on file corresponds to **Auxiliary Request 1B** which was filed at oral proceedings in first instance proceedings. Auxiliary Request 1B was already not admitted by the Opposition Division as it was considered to be late filed. Similarly, Auxiliary Request 3 as filed with the present Grounds of Appeal corresponds to **Auxiliary Request 1A** which was also filed during oral proceedings in first instance. Auxiliary Request 1A was also not admitted in the proceedings as being late filed. None of the present further requests had been submitted during first instance proceedings at all.

(4) It is noted that the Patent Proprietor does not provide either

- an explanation why these requests were filed only at this stage, nor
- an excuse why they could not have been filed earlier.

(5) However, both would be prerequisites for these requests to be admitted into the proceedings.

(6) Even further, it is notable that the present requests are not drafted in a convergent manner. Instead, several different features are introduced, e.g. Auxiliary Requests 1 and 2 comprise a delimitation regarding the sequences of eculizumab, Auxiliary Request 3 comprises a delimitation regarding the pH of the composition, but does not comprise the delimitation of the sequences of Auxiliary Request 1, Auxiliary Request 6 comprises a delimitation regarding the administration route, but comprises neither the delimitation of the Auxiliary Request 1, nor the delimitation of Auxiliary Request 3 and finally, Auxiliary Request 8 comprises a delimitation regarding a dilution to a final concentration and is again not combined with the features of either Auxiliary Request 1 or 3.

(7) Auxiliary Requests 4 and 5 in turn are combinations of Auxiliary Requests 1 and 3, or 2 and 3, respectively, but these features or feature combinations are not picked up in any of the lower-ranking Auxiliary Requests.

(8) Thus, apart from having been introduced at a late stage, without any reason for having done so and without any excuse why this has not been done any earlier, these requests also do not fulfil the requirement of a converging set of requests.

(9) It is respectively referred to the Guidelines for Examination, in particular item 3.3.12 of part H, chapter 3, which concerns admissibility of amendments and in particular the time line and structure of Auxiliary Requests. It is clearly stated in the Guidelines that

> *"If auxiliary requests are filed after the final date set in accordance with Rule 116(2), they are **usually treated as late filed** unless a summons to oral proceedings was issued as the first action.*

For late filed requests, in addition to the criteria set out in HIII, 3.3.1.1, the subject-matter of the new claims should not diverge considerably from the claims already filed. ***The requests should normally represent a convergent development****, i.e. the subject-matter of the Auxiliary Request should constitute sequential limitations in the direction of an intended invention and should not make use of different characteristics in order to branch out in different directions (T1273/04)."*

(10) The presentation of several different features in a non-converging manner amounts to "fishing for something patentable". This is particularly true in the present case. The claims have been rejected in first instance for a lack of inventive step. Thus, there is no necessity to attempt several different approaches to overcome formal issues. Consequently, the present requests do not fulfil the requirement of convergent requests and should thus for this reason alone not be admitted into the proceedings.

(11) The legal framework, the Guidelines and the case law which has been developed regarding submitting auxiliary requests, as well as documents and evidence during opposition proceedings has been developed to make sure that all parties have a fair possibility to defend their case through two instances. The case law also sets out very clearly that new auxiliary requests should be allowed only (if submitted after the deadline of Rule 116 EPC) if the amendments are ***prima facie*** **allowable**.

(12) Turning to this provision, the Opposition Division had already decided in first instance that Auxiliary Request 1A was not admissible. This request, which is identical to Auxiliary Request 3 now on file specifies that the eculizumab composition has a pH value of 7.0.

(13) The OD correctly pointed out that this feature concerns the eculizumab formulation itself and not the claimed therapeutic use to treat PNH. Thus, and in view of the fact that **D22** discloses a pH range of 6 to 8 for eculizumab solutions, the OD could not *prima facie* see how such a feature would be able to overcome the inventive step objection. Consequently, the Auxiliary Request 1A was not admitted into the proceedings.

(14) Identical considerations should apply to Auxiliary Request 3 submitted with the Grounds of Appeal.

(15) Similarly, Auxiliary Request 1B, which is identical to Auxiliary Request 1 now on file specified that eculizumab comprises a heavy chain consisting of SEQ ID NO:2 and a light chain consisting of SEQ ID NO:4.

(16) The OD correctly pointed out that the auxiliary requests were on the one hand not convergent and that arguments, which took this feature into account, had only been brought up by the Proprietor on the day of oral proceedings. Thus, there was simply no possibility whatsoever for the Opponents to react appropriately in first instance. Also, the question of the sequences of the antibody is indeed complex, as discussed by the Opposition Division in their point 2.4.3 of the decision. Filing such a request only at oral proceedings has effectively robbed the Opponents of the possibility to discuss and review this amendment in any detail in first instance proceedings. Thus, this request should clearly also not be admitted into the proceedings now.

(17) Respondent respectfully points out that the second instance is not meant to be an instance reviewing new data or new facts. Instead, it is meant to review the (legal) decision as taken in first instance. However, admitting Auxiliary Request 1 into the proceedings would clearly contravene this principle; the question of the sequences in question has not been discussed in first instance and this request is thus clearly not admissible.

(18) Incidentally, the same reasoning applies to Auxiliary Request 2 as submitted with the present Grounds of Appeal; again, the Proprietor introduces a feature related to the sequences of eculizumab. This matter has not been discussed before oral proceedings and there was no possibility for the Opponents to react to or review it appropriately.

(19) Also, it is immediately apparent from the discussion of the Proprietor in their item 6.3 of the Grounds of Appeal that this request could possibly only be considered to be originally disclosed if the person skilled in the art would be capable of understanding that the original application comprised an error regarding SEQ ID NO:4. The Proprietor needs to refer to a further document, namely **D59** - a declaration by Dr. Bruce Andrien (including four further exhibits) - to be able to support this allegation. Clearly, this cannot result in an auxiliary request, which is *prima facie* allowable. Instead it results in an auxiliary request which raises even

additional formal issues. Again, such a request should not be allowed into the proceedings at the appeal stage.

(20) As Auxiliary Requests 4 and 5 combine any of the above discussed requests with each other, they are not allowable *mutatis mutandis*, whereby the issues are even compounded by the fact that several of the amendments have been combined.

(21) The same is true for Auxiliary Request 7.

(22) In sum, none of Auxiliary Requests 1-5 and 7 should be admitted into the proceedings.

2.1.2 It is also requested to not allow document **D33** or any of documents **D54**-**D60** into the proceedings.

(23) Allowability of **D33** and **D54** was already discussed during first instance. The Opposition Division decided that **D33** and **D54** should be allowed into the proceedings. It is respectfully submitted that the Opposition Division (OD) acknowledges in their inventive step discussion that document **D33** – a document which is said to describe stability data of an eculizumab solution – is not concerned with the alleged invention described in the opposed patent. The OD correctly points out that the present patent is concerned only with a treatment of PNH; there is **no discussion of the stability of an eculizumab formulation nor any other data which would support that the present formulation would actually be particularly stable**. Thus, **D33** is completely unrelated to the present case and should thus not have been admitted into the proceedings.

(24) The same is true for the declaration **D54** which also only concerns stability data.

(25) Documents **D57**-**D60** have only been filed with the letter of appeal by the Proprietor. **D57 - D59** pertain to the issue of sequences 2 and 4; as this issue was only introduced in oral proceedings, the Opponents had no possibility to review it or discuss it in detail during first instance. Thus, these documents should not be admitted into the proceedings. It is respectfully submitted that the Proprietor clearly was aware of the issue of these sequences; **D1** and the opposed patent's data are both in the realm of

the Proprietor. Thus, if they considered this to be an important issue in their defence, they should and could have submitted both the argument, the related Auxiliary Requests as well as all of **D57**-**D59** earlier. Indeed, it appears that **D57** was already prepared on January 01, 2017 (see signature line in **D57**), while **D58** was prepared on May 11, 2017 (see signature line of **D58**). Similarly, **D59** was prepared on May 23, 2017 (see signature line of **D59**). Thus, all of these documents could (and should) have been filed with the first response of the Proprietor to the Notices of Opposition. It is respectfully noted that **D59** is also not only a declaration but comprises further documents (the **"Exhibits"**) which further complicate both the argument made therein and the amount of additional information.

(26) Interestingly, the Proprietor also does not present any excuse or explanation why these documents were filed only at this stage during second instance.

(27) **D55** are even further experimental data. They are allegedly filed in response to assertions of the Opponents during oral proceedings regarding the question of the alleged improved stability with respect to aggregation. However, the question of *"improved stability"* was raised by the Proprietor, not by the Opponents. Indeed, based on the opposed patent or its original application there was no possibility to foresee that the question of improved stability would become an issue. If the Proprietor chooses to redirect the originally stated objective of the opposed patent it is also the Proprietor's responsibility to submit all necessary supportive documents at a time where the Opponents have the opportunity to study them for both instances. Again, this is not the case here. **D55** are data which have been filed only with the Grounds of Appeal. Clearly, this again robs of the Opponents of the possibility of two instances for discussion and possible counter-arguments.

(28) **D56** are abstracts of applications cited in the original applications. They could clearly have been filed earlier. There is no explanation why they should be of *prima facie* relevance to the present case. Indeed, there is NO reference to **D56** anywhere else in the Grounds of Appeal apart from in the list of documents in item 2.1.1. **D60** pertains to salbutamol and is thus again of no relevance to the present case. In that regard: the Proprietor has argued several times that antibodies need to be considered individually and that applying results from one antibody to a different antibody is not possible (see e.g. item 3.10 of Proprietor's submission dated 21 November 2018). Salbutamol is not even an antibody. Also, this document addresses the issue of the

relevance of **D22** for the present case; **D22** was cited in the original oppositions. Any response to this document should have been submitted in first instance.

(29) None of the above documents should be allowed into the proceedings.

2.2 Main Request

(30) The Main Request of the Proprietor is to maintain the patent as granted.

2.2.1 Inventive Step, Art. 56 EPC

2.2.2.1 Priority, Art. 87 EPC

(31) The OD had decided in first instance that the present opposed patent does not enjoy its priority. We agree.

2.2.2.2 The Main Request lacks inventive step over **D1** as closest prior art[1]

(32) According to the Grounds of Appeal of the Proprietor, there are two differences between the features of claim 1 and **D1**, namely

> *(i) the sequence of eculizumab;*
> *(ii) the composition of eculizumab, namely a single-use dosage form of a sterile, preservative-free solution comprising 300 mg eculizumab at a concentration of 10 mg/ml*. (see Grounds of Appeal, item 5.1.1)

(33) While the OD adopted the interpretation of the Opponents, i.e. that claim 1 and **D1** only differ with regard to the above-mentioned second feature, the Proprietor sees a further difference, namely the above first feature, which is the sequence of eculizumab.

2.2.2.2 [1] (and over **D3** *mutatis mutandis* in view of the largely overlapping disclosure between **D1** and **D3**; we refer to our discussion of **D3** in item 3.2.2 of the original opposition brief in that regard)

(34) Before discussing the problem-solution-approach based on **D1**, we would like to address the above alleged difference regarding the sequence of eculizumab. It is, however, noted that this line of argument should also not be admitted into the proceedings. The Proprietor has raised this issue for the first time in oral proceedings which is clearly too late to be fully considered by the Opponents. Nevertheless, for the sake of argument, we submit the following.

(35) Firstly, it is respectfully referred to the language of claim 1, which reads as follows:

> *"A pharmaceutical compound 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."*

(36) The claim is clearly directed to a pharmaceutical composition which comprises the compound *"eculizumab"*. The OD correctly pointed out that the antibody eculizumab in claim 1 is not delimited by specific sequences and certainly not delimited by the specific sequences 2 and 4 of the patent.

(37) The Proprietor however seems to imply that the antibody eculizumab is indeed defined further by specific sequences, namely SEQ ID NO:2 for the heavy chain and SEQ ID NO:4 for the light chain. This is incorrect for several reasons.

(38) a) The antibody eculizumab cannot be defined by SEQ ID NO:2 and 4 as SEQ ID NO:4 refers to the entire light chain, including the signal peptide. As argued extensively by the Patent Proprietor himself, **the antibody *"eculizumab"* however comprises a light chain without this additional signal peptide**. Thus, for this reason alone, the antibody *"eculizumab"* cannot be defined by SEQ ID NO:2 and 4.

(39) When interpreting the term "eculizumab" in the light of the specification - which of course would be the correct approach for shedding light on terms used in the claims - it is immediately clear that eculizumab is not delimited to an antibody comprising specific sequences.

(40) It is respectfully referred to paragraph [0011] of the opposed patent. In the second sentence it is stated that

"*The antibody that binds C5 or an active antibody fragment thereof is eculizumab*".

(41) Thus, the patent clearly defines eculizumab as an antibody "*that binds C5*". The term "*eculizumab*" is thus not further delimited according to the opposed patent itself.

(42) Similarly, paragraph [0016] of the opposed patent states:

"S*aid antibody binds C5 and is eculizumab".*

(43) **Again, the antibody is thus not further delimited then merely by its binding ability to C5.**

(44) Indeed, the paragraph serving as basis for the dosage form as claimed in claim 1 also refers to "*said antibody that binds C5*"; please see paragraph [0017], first sentence thereof of the opposed patent.

(45) When turning to the passage which deals with specific sequences, it is even more clear that the term "*eculizumab*" is not delimited to the specific sequences of SEQ ID NO:2 and 4. This passage is paragraph [0018] of the opposed patent.

(46) The passage starts with the words "*in certain embodiments*". This terminology indicates that the following description will refer only to some embodiments of e.g. preferred antibodies. It is then stated that "*the antibody that binds C5*", i.e. eculizumab, see above, "*comprises a heavy chain variable region, wherein the heavy chain variable region comprises **one or more** CDR regions having amino acid sequence selected form 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 amino acid sequence selected from the group consisting of SEQ ID NO: 8, SEQ ID NO: 9 **or** SEQ ID NO: 10."* (emphasis added)

(47) Thus, eculizumab is defined in some embodiments of the opposed patent as comprising specific CDRs; a CDR based definition is of course a much broader definition than the more delimited definition by way of the specific heavy chain and the light chain of SEQ ID Nos 2 and 4. Even further, the definition allows for only one

of these CDRs being present, as long as the resultant antibody binds C5. Only in "*certain embodiments*" the antibody that binds C5 is defined to comprise a heavy and a light chain wherein the heavy chain consists of SEQ ID NO: 2 and the light chain consists of SEQ ID NO: 4 (last sentence of paragraph [0018]).

(48) The above is again further supported by paragraph [0030], third sentence which emphasizes that eculizumab is used, "*which binds to or otherwise blocks the generation and/or activity of C5*"

(49) A further definition of eculizumab and what is meant to fall under this definition could potentially be taken from paragraph [0054]. Here, it is referred to U.S. Patent 6,355,245, which is present as document **D17** in the present proceedings. However, while **D17** does clearly disclose the antibody H5G1.1 (which is for example also used in **D1**) and the patent itself links it to the term "*eculizumab*", there are no explicit indications as to which sequences are provided or used in **D17**, as also correctly acknowledged by the OD. Indeed, the Proprietor supports this notion, see item 5.5.3 subsequent of their Grounds of Appeal:

> "*Neither D17 nor D13*[2] *mentions "eculizumab". D17 and D13 describe the cloning of murine antibody 5G1.1 and the construction of humanised variable regions and humanised antibody fragments. … As noted by the Opposition Division, D17 does not give a complete sequence of a humanised 5G1 antibody. …. D13 also fails to provide complete heavy and light chain sequences of a full length antibody*."

(50) Thus, paragraph [0054] is not suitable to further delimit the meaning of eculizumab, which the patent otherwise defines quite broadly, see above. Instead, at best it would add to the assumption that eculizumab does not have a clear and well defined meaning delimited to specific sequences.

(51) A final mentioning of eculizumab can be found in paragraph [0074] of the opposed patent. Here, the Applicants explained which antibody was used in the trial which constitutes the example of the opposed patent. Firstly, of course this does not delimit the overall meaning of eculizumab as used in claim 1 and defined throughout

[2] D13 (as D17) is cited in D1.

the general specification. Secondly, the only further indication of what eculizumab is meant to be is that it is the antibody "*Soliris™,* of *"Alexion Pharmaceuticals, Inc.*". However, in the proprietor's own submission, there was no information on Soliris™ available before the application date of the present patent; please refer to item 5.5.8 of the Grounds of Appeal in that regard. Thus, this indication does not add any further delimitation of what is meant by *"eculizumab"* in the context of the opposed patent and in particular in claim 1.

(52) The reference to "*eculizumab*" in the sequence list also cannot be used to delimit the meaning of "*eculizumab*" in the context of claim 1. This is because the patent as such provides a broader definition and the person skilled in the art would understand that the sequences of the sequence list are only representative of a preferred embodiment. Again, it is respectively submitted that even this preferred embodiment does not correctly represent the final antibody as the final antibody would not comprise the entirety of SEQ ID NO: 4.

(53) For these reasons, **including the sequence information for eculizumab into the features differentiating claim 1 of the Main Request from D1 is incorrect.**

(54) The same reasoning also applies to the earlier attempt of the Proprietor to introduce an artificial difference between the term "eculizumab" as used in claim 1 of the opposed patent and the disclosure of **D1**. This difference was said to reside in the IgG subtype of eculizumab. The Proprietor points to **D13** (as cited in **D1**), which does comprise a reference to the production of an antibody with an IgG4 subtype (page 1399, right hand column of **D13**, second paragraph, lines 15 – 25). However, as the Proprietor points out correctly in item 5.5.3 of their Grounds of Appeal "*Neither D17 nor D13 mentions "eculizumab*". Thus, this argument must fall short for this reason alone. Even further: as shown above in detail, the term "eculizumab" as used in claim 1 of the opposed patent is not delimited to a specific IgG subtype, nor to any specific sequence. Thus, claim 1 of the opposed patent would encompass the antibody described in **D13** as well. Even further, the antibodies described in **D1** are not delimited to the one specific antibody as described in **D13** but refer generally to anti-C5 antibodies (see e.g. [0012), third sentence, or [0037], second sentence of **D1**). Also, as acknowledged by the Proprietor and the OD, the **D1** disclosure also refers to **D17**, next to **D13** for specific antibodies. **D17** clearly points out that ".. *constant domains from IgGs of various subtypes, …* " could be used for the construction of their recombinant antibodies. Thus, **D1** in and out of itself (as well as when taking

into consideration either one or both of **D13** or **17**) clearly discloses an antibody with any possible IgG domain.

(55) It is of further interest that the Proprietor needs to refer to (late filed!) declaration **D57**, where it is explained that the commercially marketed eculizumab antibody-variant has a "*unique, non-naturally occurring, protein engineered heavy … and light chain*". This disclosure is not part of the opposed patent. It thus cannot serve to distinguish claim 1 of the opposed patent from the prior art. As the Proprietor argues extensively in items 5.5.7 subsequent of the Grounds of Appeal (referring to declarations **D57** and **D58**) the commercial eculizumab antibody was NOT available to the public. Thus, any information on this specific antibody could only have come from the opposed patent (as to the definition of "eculizumab" in the claim). We have discussed this definition extensively above; neither of **D57** nor **D58** can change this situation.

(56) Our following problem solution approach will thus be based on the correct interpretation of claim 1 and will focus on the *"feature ii"* as identified by the patent Proprietor in the Grounds of Appeal.

(57) If the Board of Appeal should come to a different conclusion with regard to the above, we refer to our discussion of inventive step of Auxiliary Request 1, which also takes into account the feature of SEQ ID Nos 2 and 4.

Disclosure of D1

(58) The disclosure of **D1** relates to a method of treating ***PNH*** in mammals (see for example, [0012], [0036]), in particular in human ***patients*** afflicted with this disease (see [0067]; Examples).

(59) The Examples report on the implementation of a clinical trial to evaluate the effects of an anti-C5 antibody on PNH and symptoms associated therewith ([0081]). The anti-C5 antibody used was **eculizumab** (see [0082]; Figures).

(60) "***Eculizumab***" is a designation for an anti-C5 antibody "***h5G1.1-mAb***" (see [0012], penultimate line; [0052]). 'As has been argued extensively above, this antibody complies with the *"eculizumab"* definition of the opposed patent. According to

section [0052] of **D1**, the h5G1.1-mAb, i.e. eculizumab, and methods for manufacturing it were already known from the prior art:

> *Particularly useful anti-C5-antibodies are **h5G1.1-mAb**, h5G1.1-scFv and other functional fragments of h5G1.1. **Methods for the preparation of h5G1.1-mAb**, h5G1.1-scFv and other functional fragments of h5G1.1 **are described in U.S. Pat. No. 6,355,245** and "Inhibition of Complement Activity by Humanized Anti-C5 Antibody and Single Chains Fv", **Thomas et al.,** Molecular Immunology, Vol. 33, No. 17/18, pages 1389-1401, **1996**, the disclosure of which are **incorporated herein in their entirety by this reference**. The **antibody h5G1.1-mAb is currently undergoing clinical trials under the trade-name eculizumab**.*
> (lower part of [0052]; emphasis added; the cited US 6,355,245 is **D17** of the present proceedings and Thomas et al. is **D13** of the present proceedings)

(61) The mode of administration of eculizumab to a patient suggested by **D1** is, *inter alia*, "*via intravenous infusion*" ([0060]) in a variety of "*unit dosage forms*" ([0058]). The actual dosage regimen used in said clinical trial is described in [0082]:

> *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.*

(62) In summary, **D1** discloses the treatment of PNH patients with a pharmaceutical composition comprising eculizumab, said pharmaceutical composition being administered via intravenous infusion. The formulation must be sterile: see 2nd sentence of [0062] of **D1**. The solution can be free of preservatives: see last sentence of [0062] of **D1**. This definition of providing an eculizumab formulation either with or without preservative is identical to the language of the opposed patent which equally envisages both possibilities side-by-side:

> "*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 composition of the application*."
([0064] of opposed patent, emphasis added)

(63) As an aside to the question of the feature *"preservative-free"*: As we have pointed out above, **D1** comprises clear disclosure that the formulation of the eculizumab antibody can be preservative-free. However, just for the sake of good order, it is additionally referred to the general knowledge as represented for example by either **D19** or **D29**. **D19** is a pre-published publication of the European Agency for the Evaluation of Medicinal Products and is a guidance on excipients, antioxidants and antimicrobial preservatives in the dossier for application for marketing authorization of a medicinal product. In the chapter on preservatives, starting on page 8/9 of **D19** it becomes clear that multidose preparations and eyedrops are those solutions which are in particular at risk of contamination. See also last sentence on the last page of **D19**. It is self-evident that single-use formulations are not associated with a similar risk of contamination and thus, addition of preservatives is usually not necessary, which is well-known.

(64) Similar disclosure is available from column 7, lines 13 subsequent of **D29**.

(65) In any case: the above cited disclosure from the opposed patent in paragraph [0064] clearly shows that whether or not a preservative is added is arbitrary and is not associated with any particular technical effect.

Objective Technical Problem

(66) As outlined above, **D1** discloses a pharmaceutical composition for use in treating a patient afflicted with PNH in the clinical study of the Examples. The active ingredient is eculizumab.

(67) Since the eculizumab comprising composition of **D1** is to be administered via intravenous infusion, it must be constituted as an aqueous solution, and must be sterile.

(68) As compared to **D1**, granted claim 1 provides the following further features in an explicit way[3]:

- the **total amount** of eculizumab in the single-use dosage form is **300 mg,**
- the eculizumab **concentration** is **10 mg / ml,**
- a **volume** of **30 m**l.

No technical effect

(69) No technical effects or advantages whatsoever in terms of, for example, stability of the liquid formulation or shelf-life, are disclosed or even merely suggested in the patent specification. No comparative experiments with other administration forms had been performed. The patent also comprises no data on stability of such formulations whatsoever.

(70) Notably, the section "EXEMPLIFICATION" in the opposed patent, which describes the "TRUMPH" clinical study, only specifies the total amount of eculizumab (600 mg or 900 mg) administered to the patients via intravenous infusion (see [0074]) but is equally silent as **D1** on the features above. Thus, it remains unclear whether the eculizumab formulation as claimed was actually used in the Examples of the opposed patent.

(71) It is also of note that the opposed patent's examples are identical regarding the dosage as the examples of **D1**.

(72) Please compare here paragraph [0074] of the opposed patent:

- Compound used: eculizumab
- 600 mg eculizumab via intravenous infusion every 7 days for 4 doses
- followed by 900 mg eculizumab via intravenous infusion 7 days later
- followed by a maintenance dose of 900 mg eculizumab via IV infusion every 14 days for a total of 26 weeks of treatment.

[3] We will show below that the dosing regime is 100% identical between **D1** and the opposed patent; the only difference being that there is no general indication in **D1** on amounts/concentration in the single-use dosage forms.

(73) Compare this with the respective disclosure in **D1**, Table 1 on page 9:

- Compound used: eculizumab (an anti-C5 antibody according to paragraph [0082] of **D1**)
- 600 mg eculizumab via intravenous infusion every 7 days for 4 doses
- followed by 900 mg eculizumab via intravenous infusion 7 days later
- followed by a maintenance dose of 900 mg eculizumab via IV infusion every 14 days for a total of two years of treatment.

(74) Thus, the examples presented in **D1** are identical to the examples as presented in the opposed patent.

(75) There is clearly no scientific contribution whatsoever which was exemplified in the opposed patent compared to the disclosure already presented in document **D1**.

(76) The objective technical problem underlying the opposed patent is therefore to provide a – presumably - alternative pharmaceutical composition comprising eculizumab for use in the clinical studies described in **D1** for the same compound and with the same dosing regimen.

(77) Said composition is characterized in terms of the absolute amount and concentration of eculizumab. The Proprietor argues that the opposed patent actually provides for an improved pharmaceutical formulation. Respondent disagrees. The above approach is correct and based on the data and language provided in the patent *per se*[4].

(78) This absolute amount and concentration of eculizumab are however obvious alternatives in view of the disclosure of **D1** itself. As described above, the clinical trial of **D1** uses the amount of either 600 mg eculizumab or 900 mg eculizumab via IV infusion (i.e. the identical dosing regimen as in the example of the opposed patent).

(79) Thus, if the person skilled in the art on the basis of the disclosure of **D1** would want to provide a single use dosage form, the amount of 300 mg would be the exact

[4] The approach of the Proprietor will nevertheless be discussed below in detail.

amount which would be most suitable for both the 600 and the 900 mg total amount. This leaves one actual feature which is not derivable from **D1** in an explicit manner, namely the antibody concentration of 10 mg/ml.

(80) **D1** in combination with general knowledge

(81) However, this antibody concentration is the most frequently used concentration in commercial antibody formulations and is thus part of the common general knowledge.

(82) To support this, Respondent refers to document **D20**.

(83) **D20** reflects on pharmaceutical antibody formulations that have been approved by the health authorities before the filing date of the opposed patent. It is a **review article** and therefore belongs to the common general knowledge in the sense of T 309/88. As stated in the Abstract, by 2006 more than 23 monoclonal antibodies have entered the US market. An overview on the properties of these commercial antibody products, such as administration route or antibody concentration as well as pH, is given in **Table 1** of **D20**.

(84) It is readily presumed that a formulation specialist would have consulted **D20** for guidance when tasked with the manufacture of a therapeutic antibody formulation.

(85) Table 1 of **D20** lists **23 commercial therapeutic products** comprising authorized antibodies. The antibody concentration in the formulation for each product is given in the column headed "*Mab Conc*". One can reasonably presume that a skilled person - commissioned to prepare a therapeutic formulation for a new antibody - would have oriented himself on existing commercial antibody formulations, in particular with respect of such a mandatory parameter as the antibody concentration, all the more so because the number of approved antibodies at the priority date was only 23, i.e. a fairly manageable amount.

(86) The Proprietor argues regarding **D20** that this document only shows those antibody products which have been commercially approved. The Proprietor correctly points out that there are many more antibody products which have never reached the market. However, this actually makes the disclosure of **D20** even more relevant: it

summarizes exactly those antibody formulations which are successful, stable and have been approved for commercialization and would thus be a strong indicator for the person skilled in the art which concentration would have a high likelihood of success to be suitable and stable.

(87) In the table of **D31**, the antibodies of Table 1 of **D20** were grouped according three concentration ranges. In case the antibody concentration was exactly 10 mg/ml, the box in the last column (10 mg/ml) was checked.

(88) Since the antibody formulation of the present invention is not limited to any mode of administration, also routes other than intravenous administration were considered. Moreover, since the formulation according to claim 1 can also be reconstructed from a lyophilized powder formulation, such commercial products were likewise included.

(89) As one can take from column 5 of **D1**, 20 out of 23 therapeutic antibody formulations **(87%)** have a concentration within the range of 0.1 to 50 mg/ml. Most remarkably, the number of commercial antibody formulations having a concentration of exactly **10 mg/ml** was still as high as **22%** (5 out of 23)!

(90) The individual antibody concentrations of the respective antibodies vary within the above ranges, but it is immediately apparent that 1**0 mg/ml is the most commonly used concentration** (5 formulations, versus 2 formulations with 0.5 mg/ml or 2 mg/ml, respectively).

(91) The clear and straightforward preference for an antibody concentration of 10 mg/ml becomes even more manifest if only antibody formulations for intravenous administration (18 antibody products) are considered. According to **D32**, 18 out of 18 therapeutic antibody formulations **(100%)** have a concentration within the range of 0.1 to 50 mg / ml. The number of antibody formulations having a concentration of exactly **10 mg/ml** is still **22%** (4 out of 18). The concentration of 10 mg/ml is thus – based on common general knowledge – the most straightforward and obvious choice for a formulation scientist aiming at providing an antibody formulation.

Total volume 30 ml

(92) As explained above, the volume of the composition inherently follows from the absolute amount (300 mg) and the concentration of the active ingredient in the solution (10 mg/ml). Since both features are obvious as demonstrated above, also feature 5 relating to the volume of 30 ml is also obvious (300 mg/(10 mg/ml) = 30 ml).

(93) The Main Request is thus not inventive in view of **D1** in combination with general knowledge, as represented by **D20**, **D31** or **D32**.

D1 in combination with **D22**

(94) Claim 1 of the Main Request is also not inventive in view of a combination of **D1** with **D22**.

(95) **D22** explicitly concerns formulations of eculizumab antibodies: see summary of **D22**, in paragraph [0004] thereof. The third sentence of paragraph [0004] refers to anti-C5 antibodies as described in US patent number 6,355,245, i.e. **D17** (the same document as cited as basis for eculizumab antibodies as in the opposed patent) and two sentences further down it is referred explicitly to preferred embodiments which employ either pexelizumab or eculizumab as the antibody therapeutic. Eculizumab is mentioned throughout **D22** as the preferred antibody.

(96) Thus, any argument brought forward by the Proprietor regarding non-comparability of formulations for different antibodies cannot apply to document **D22**. As **D22** actually describes an eculizumab formulation, the person skilled in the art not only could but would have turned to this document.

(97) Paragraph [0006] states that the antibody should be formulated in a composition which is *"suitable for aerosolization"*.

(98) Similarly, the antibody can also be formulated in a composition *"suitable for nebulization"* (see second sentence of paragraph [0007] of **D22)**. **D22** points out in paragraph [0013] that an example of a formulation which is suitable for

aerosolization or nebulization of an antibody is in physiologic osmolarity and at a suitable pH, whereby the suitable pH is said to be pH 6-8.

(99) It is respectfully referred to paragraph [0062], middle thereof in **D22** which states

> *"In either type of nebulizer, the **drug is usually contained in solution** in the liquid in the nebulizer and so the droplets being produced **contain drug in solution**."* (emphasis added)

(100) This is in particular noteworthy in view of the indication of the Proprietor in the Grounds of Appeal 5.6.5 where they state in their third sentence

> *"Therefore, the lack of any mention that the formulation is a solution is significant in the context of a formulation for nebulization."*

(101) It is respectfully submitted that this is simply incorrect. It is very clear from the above citation in paragraph [0062] that the drug is **usually** contained **in solution**. Thus, while there are exceptions where the drug is contained in small particles suspended in water, the usual and typical formulation which is described in **D22** is a drug in solution. The assessment of the Opposition Division in that regard is thus entirely correct; indeed, it would be unreasonable to assume that the drug would be in a slurry or suspension. This also overcomes the argument of the Proprietor in their item 5.6.9 where they again base their defense against **D22** on the assumption that *"**D22** provides no indication that the formulations of eculizumab referred to in paragraphs [0171]-[0172] are solutions"*.

(102) Indeed, it must be emphasized once more that the usual formulations described in document **D22** are solutions.

(103) **D22** is also explicit as to preferred concentration ranges. Paragraph [0067], last sentence refers to preferred concentration ranges from 1 mg/ml to 200 mg/ml and even additionally indicates that the antibody can be stable in this range. Clearly more preferred concentrations are then described in paragraphs [0170] - [0172] and Figure 10 of the **D22** disclosure. Formulation 1 here comprises the monoclonal antibody with 1-30 mg/ml. Thus, the disclosure points the person skilled in the art in an

obvious manner to using the 10 mg/ml concentration for the eculizumab antibody in solution.

(104) The Proprietor argues that the person skilled in the art had no expectation of success for 10 mg/ml as in an IV administration mode a higher amount of antibody would be expected to actually be active in the patient than in an inhalation administration mode. However, on the one hand, the Main Request is not delimited to intravenous administration. On the other hand, the 10 mg/ml is at the lower end of the preferred range in **D22** and thus would take this into account. Even further, we have shown above that the general knowledge supports that a 10 mg/ml concentration is the most suitable and preferred one for antibodies in general, and for antibodies designed for intravenous administration.

(105) The Proprietor also refers to document **D60; D60** should here not be taken into account. **D60** is clearly late filed, as has been shown above. However, even if taken into account, **D60** pertains to salbutamol which is not even an antibody but a small molecule. Whatever is true for a small molecule compound certainly cannot be extrapolated to the present antibody. In particular, **D1** and **D22** concern the exact same antibody, namely eculizumab, which is also claimed in the patent and are thus highly relevant for the person skilled in the art who is looking for an alternative formulation to the one explicitly already described in **D1**.

(106) Any disclosure in **D60**, concerned with salbutamol, would certainly not deter the person skilled in the art to take into account the disclosure of explicit eculizumab formulations when looking for such an alternative formulation.

(107) The Proprietor finally points out that many antibodies cannot be formulated as a liquid at all, since they lack the required stability. However, it is entirely clear, even inherent, from the disclosure of both **D1** and **D22** that eculizumab is stable in solution. **D1** describes a clinical trial carried out with an eculizumab formulation which is administered IV; thus, the antibody most certainly must have been available in solution and as the Proprietor correctly points out – certainly manufacture of this antibody did not occur at the bedside of the patient. Thus, the two arguments presented by the Proprietor regarding **D20** must fall short for the above reasons.

(108) **D1** in combination with **D29**

(109) **D29** is concerned with the provision of stable aqueous pharmaceutical formulations comprising an antibody.

(110) The antibody concentration in the formulations of **D29** is typically from about 0.1 to about 50 mg/ml (see e.g. claim 13). Preferred antibody concentrations are mentioned in column 22, lines 13 to 17 of the specification:

> *From about 0.1 mg/ml to about 50 mg/ml, preferably, from about 0.5 mg/ml to about 25 mg ml, and **most preferably** from 2 mg/ml to about **10 mg/ml** is an exemplary antibody concentration in the formulation (cf. col. 22, l.13-17).*
> (emphasis added)

(111) The antibody concentration of "***10 mg/ml***" is **expressly** mentioned in **D29** as belonging to the **most preferred** concentration range.

(112) It was obvious to the skilled person with a reasonable expectation of success that the antibody concentrations mentioned in **D29** would be also applicable for a pharmaceutical eculizumab formulation. The antibody concentration of 10 mg/ml is therefore an entirely obvious choice.

(113) Clearly, the person skilled in the art looking for a preferred antibody concentration for the formulation of **D1** would have arrived at 10 mg/ml in an obvious way, based on the disclosure in **D29**.

(114) The skilled person would conclude from **D29** that the antibody concentrations suggested in **D29**, in particular 10 mg/ml, would be generally applicable for many different antibodies. In fact, every 5th approved antibody that was commercialized before the priority date of the opposed patent, was successfully used at exactly the same concentration as claimed in the opposed patent, i.e. 10 mg/ml, as has been shown above.

(115) It was therefore obvious for the skilled person to try out this concentration also for eculizumab with a reasonable expectation of success. The selection of 10 mg/ml does not involve an inventive step.

(116) As regards the argument of the Proprietor that **D29** is *"primarily related to formulations of anti-CD18 and anti-CD20 antibodies"*, this is indeed correct. However, the proposals for specific formulation ranges are made not in the context of specific antibodies but for antibody formulations in general.

(117) Thus, this argument does not deter from the applicability of **D29**.

(118) As an aside: as an antigen of special interest **D29** mentions the **CD20 surface antigen** which can be targeted for treating B cell lymphomas (cf. col.2, l.59-61). Several antibodies against CD20 were known at the filing date of **D29**, including the chimeric antibody termed "***C2B8***" (cf. col.2, lines 14-18 & reference to US 5,736,137 by Idec Pharmaceutical Corporation). Idec's C2B8 antibody is also known by the INN (international nonproprietary name) "***rituximab***", see for example Maloney et al. 1997 (**D30**; title).

The antibody product "Rituxan" comprising rituximab is listed among the 23 approved pharmaceutical antibody formulation of **D20**. According to Table 1, no 16, Rituxan comprises "10 mg/ml" of antibody in a solution of intravenous administration. **Preferred antibodies of D29 are thus also commercially used at a concentration of 10 mg/ml.**

D1 in combination with **D21**

(119) In principle, similar arguments apply for the combination of **D1** with **D21**. **D21** is – as **D20** – a document belonging to the common general knowledge of the person skilled in the art. It reviews the formulation of in particular antibody therapeutics. The Proprietor seems to argue that **D21** is not applicable as the *"majority of work on antibody formulation has been carried out on IgG1 antibodies"*. The Proprietor once more alleges that eculizumab is not such an IgG1 antibody. We refer respectfully to

our above discussion of the definition of eculizumab which clearly does not exclude IgG1 antibodies; for that reason alone this argument must fall short.

(120) This is similarly true for the allegation that *"eculizumab has a unique engineered structure"* and as such antibodies were not available at the publication date of **D21**, **D21** would allegedly not be applicable. It is unclear why an engineered antibody like eculizumab would be behaving particularly different from the many different antibodies as reviewed in **D21**. This assumption seems to be scientifically unreasonable.

(121) In sum, claim 1 of the Main Request is entirely obvious in view of **D1**, **D1** in combination with the general knowledge, or **D1** in combination with either one of **D22**, **D20**, **D21** or **D29**.

The reformulation of the technical problem

(122) The Proprietor emphasizes in the Grounds of Appeal that the technical object should be formulated as the provision of an improved formulation which has a very good stability. The advantage is said to be that the formulation *"avoids the need for reconstitution prior to IV administration"*. This seems to be an inherent feature of every solution which has not been lyophilized. Clearly, the disclosure of **D1** encompasses this situation; as we have shown in detail above, **D1** also concerns in particular antibody, namely eculizumab, solutions.

(123) When determining what has been shown and what has not been shown or which advantages have been supported by an alleged invention, it is imperative to turn to the actual patent as such. Thus, the Respondent now provides an overview of what has been shown in the opposed patent and notably, what has not been shown.

(124) When turning to the opposed patent, the background part is entirely concerned with a discussion of the treatment options which were available for the eculizumab monoclonal antibody. The last sentence of this background section reads

> *"However, this unblinded study involved a small number of patients with no control arm and without protocol driven transfusion standards."*[5]

(125) Thus, the person skilled in the art starting to read the disclosure of the opposed patent understands that the present patent is meant to overcome issues allegedly present in the study of **D1** which issues are related to the actual treatment of PNH.

(126) Under the summary part of opposed patent it is then explained that **the present** pivotal phase 3 study (i.e. the examples of the opposed patent) evaluated the effect of eculizumab on hemoglobin levels and transfusion requirements during six months of treatment in a cohort of PNH patients. In particular, measures of intravascular hemolyzes and quality of life were also assessed. The opposed patent explicitly states in the last sentence of paragraph [0005]:

> *"This is the first placebo control study of a PNH patient population to control hemolysis and to differentiate between the effects due to hemolysis and the effect due to anemia."*

(127) Paragraph [0006] then summarizes the discoveries of the opposed patent as follows:

> *"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 the transfusion."*

(128) The paragraph continues by stating which aspects fall under improvements in quality of life, none of which refers to the stability of the formulation or any measure of stability of formulation. Further disclosure then continues by discussing these particular aspects and the assays which have actually been carried out. One such assay is for example the so-called FACIT-Fatigue score, as explained in paragraph [0013] to [0014] or the EORTC QLQ-C30 score, as explained in paragraph [0015].

(129) While paragraph [0017] does describe the eculizumab antibody *per se*, further disclosure is directed exclusively to the treatment aspect. See for example the

[5] This is actually the study described in **D1** and **D3**, respectively.

paragraph under the heading "Overview", namely paragraph [0030], which is again related exclusively to treatment aspects. Further paragraphs concern the complement system (paragraph [0031] to [0043]), or measures of quality of life, see paragraphs [0044] to [0052], as well as a chapter on inhibitors of the compliment cascades, paragraphs [0053] to [0055]. A chapter on method of treatments is summarized in paragraph [0056].

(130) Chapter VII is then directed to pharmaceutical formulations and uses and while mentioning the formulation which is currently claimed in claim 1, it is of interest that paragraph [0063] actually refers to quite a few further formulations which are considered by the applicants themselves in their Grounds of Appeal as "*particularly useful for antibody based therapeutic agents*". It is of interest that the Proprietor provides an overview of the applications recited in paragraph [0063] which is enclosed in the Proprietor's submission as **D56**. Should **D56** admitted into the proceedings we already point out preemptively that these applications seem to be directed mostly to different antibodies then eculizumab; however, the Proprietor clearly states in their own opposed patent that these are formulations "particularly useful" for antibody based therapeutics in general.

(131) Even in this chapter on pharmaceutical formulations and uses thereof there is simply no reference at all to particularly preferred formulations which would enhance stability of a formulation or in particular of the eculizumab formulation. This passage ends the general part of the opposed patent. The remainder of the patent is the exemplification and concerns the TRIUMPH trial which is exclusively concerned with measures of clinical efficacy, and safety assessments. Clinical efficacy is described in detail in paragraphs [0086] subsequent and pertains to stabilization of hemoglobin levels and reduction of PRBC units, with the results being summarized in Table 1. Improvements of quality in life measures are summarized in Table 2 and concern global health status and several functional and symptomatic measurements. Finally, this study also looked at safety.

(132) However, the **examples** comprise no data at all on stability of a formulation. The **general part** comprises no reference to stability at all. The **discussion** part, following the exemplification, again focuses entirely on treatment and comprises no reference to stability of the formulation.

(133) While we acknowledge that a problem can under certain circumstances be reformulated based on the available prior art and that this is established in case law, we deny that a problem can be formulated without any concomitant and general indication in the body of the original application and without any concomitant data at all in the patent in question.

(134) The Guidelines in G VII, 11 state that

> *"Care must be taken, however, whenever new effects in support of inventive step are referred to. Such new effects can only be taken into account if they are implied by or at least related to the technical problem initially suggested in the originally filed application…"*

(135) We respectfully submit that a patent, which is entirely silent on the issue of stability of a formulation and deals exclusively with treatment options, cannot be reformulated to suddenly "imply" the stability question. Certainly, it is true that a formulation which is very unstable will be less active over time. We do not wish to deny this. However,

- in the absence of any indication in the opposed patent as to this issue,
- and in the absence of any indication in the opposed patent as to whether the eculizumab which was actually used in the trial had been stored for a day, a week, a month or 6 months or even 2 years,
- further in the absence of any indication whether the eculizumab had been stored at 4°, 25° or 40°,
- further in the absence of any indication of whether or not that storage influenced the activity of the eculizumab tested in the exemplification of the opposed patent,

(136) the patent cannot provide any support of the stability of the pharmaceutical formulation and does not comprise any indication of whether or not it was particularly stable or not particularly stable; this is undeniably true even if taking implicit disclosure into account.

(137) The Proprietor argues that the formulation was certainly not made at the bedside of the patient, and thus at least some short storage must have occurred. Nevertheless,

the patent does not say so; the person skilled in the art reading the opposed patent has no idea how long the formulation was stored and under which conditions. There is no indication that this storage was of any relevance whatsoever, and whether the data obtained in the study of the opposed patent could have e.g. been even better had the antibody been stored for a shorter period of time, or in a different formulation. There is also no indication that there was any difference in the preparation of the formulation compared to the one which was actually used in **D1** (please recall – the **D1** study is used as the starting point for the opposed patent, see citation above; the only add-on which is described in the opposed patent *vis-à-vis* this study is said to be in the better evaluation of possible treatment results; with all probability, the **D1** study was carried out with the exact same formulation as is described in the opposed patent). The person skilled in the art has no reason to assume that the opposed patent is concerned with stability of a formulation; he or she has also no reason to assume that the formulation changed over **D1**. Stability is absolutely not implied in the language of the opposed patent.

(138) To support the above, Respondents herewith submit T 386/89 which is cited in the above Guidelines as a basic decision regarding the question of whether or not a new technical effect can lead to a reformulated technical problem.

(139) We refer in particular to item 4.1 of this decision. Here, it becomes clear that the original application in the T 386/89 case had focused on the achievement of material savings; the Proprietor then attempted to argue that the technical problem was now however to be seen in the enhancement of the fatigue life of a wheel.

(140) While the Board in the above case admitted that the feature which was said to lead to this enhancement of the fatigue life was clearly described (similar to the present situation, where concentration and amount of the formulation are indeed clearly described) the effect was however not self-evident: the Proprietor indeed argued that the increase in fatigue life was surprising (and thus inventive). This again is similar to the present case where the Proprietor argues that the enhancement in stability was surprising and not obvious. The Board of Appeal in the above case also pointed to the fact that there was no disclosure or suggestion in the originally filed application that the feature in question was associated with increasing the fatigue life of the wheel (which is again applicable to the present case where there is no

disclosure or suggestion in the originally filed application that the amount or concentration of the antibody are linked in any way to stability of the formulation).

(141) The Board in the above case then discussed decision T 184/82 where the Board allowed a redefinition of the problem to an extent that the skilled person could recognize the same as implied or related to the problem initially suggested, which interpretation is also adopted here by the present Proprietor. Similarly, decision T 13/84 is taken into account. The Board in the above case however clearly states that only as long as the problem can be clearly deduced by the skilled person from the application as originally filed when considered in relation to the nearest prior art would it be allowable to reformulate the technical problem. However, there is no way the person skilled in the art could have "clearly deduced" from the application as filed in view of the closest prior art **D1** that the opposed patent actually provided a particularly stable formulation, as alleged by the Proprietor.

(142) Thus, the same conclusion as the Board reached in the above case should be reached here; we refer respectfully to the last paragraph on page 9 where the Board concluded that applying the above principles the alleged unexpected effect (in that case the improved fatigue life, in this case the improved stability) is not deducible form the application as originally filed and thus cannot be taken into account when determining the problem underlying the invention for the purpose of assessing the issue of inventive step.

(143) As pointed out above, all documents related to this alleged improved stability are thus entirely irrelevant to the present case and should not be admitted into the proceedings.

(144) Should the Board of Appeal nevertheless take into account the additionally submitted documents, we submit the following:

D33 merely confirms common knowledge of skilled person before the filing date

(145) In **D33**, Dr. Miano provides data, allegedly demonstrating the improved stability of an eculizumab formulation at 10 mg/ml compared to 30 mg/ml. As outlined above, the new experiments should not be admitted into the proceedings and therefore not be taken into account for the assessment of inventive step. However, even if the

Opposition Division considers the data relevant, they are not sufficient to support inventive step. To explain in more detail:

(146) It was common general knowledge before the filing date of the opposed patent that increasing the concentration of antibodies increases the aggregation tendency of the protein, and thus decreases its stability. In fact, there is an almost linear relationship between an increase in concentration and aggregate formation. This is reflected, e.g., by the review article **D20**:

> *"Increasing the concentration of antibodies often increases the aggregation tendency of the protein. It was demonstrated that increasing the IgG1 concentration (in 10 mM citric acid, 100 mM NaCl pH 5.5) from 2.7 mg/mL to 50 mg/mL almost linearly increased the Nephelometric Unit (NU) from 2 to 40.[52]"* (p. 9, left col., 3rd para. of **D20**)

(147) Hence, it was entirely predictable that an antibody composition formulated at 10 mg/ml shows less protein aggregation (i.e. a higher monomer content) than a composition formulated at 30 mg/ml. The data presented in **D33** merely confirm this trivial fact, which would have been obvious to the skilled person before the filing date of the opposed patent.

(148) Even further: having the commercial requirement of a high monomer content in mind and knowing that aggregate formation of antibodies increases with increasing concentration, it was a routine task for the skilled person to test a variety of antibody concentrations and determine the storage time after which the monomer content reaches a predetermined threshold level. **D35** (which summarizes the table of **D20** and **D21**) shows that antibody concentrations between 0.5 mg/ml and 50 mg/ml were routinely used for pharmaceutical formulations before the filing date of the opposed patent, and thus would have been included in the stability tests conducted by the skilled person.

(149) In **D33**, Dr. Miano merely describes the exact kind of stability studies that would have been routine to the skilled person.

(150) Auxiliary it is referred to T1241/03. In the case underlying T1241/03, the board considered the problem underlying the patent in suit to be the provision of an

aqueous formulation of human growth hormone which remains stable during long-term storage. The Proprietor argued that that a skilled person, knowing from the prior art that the development of stable aqueous protein formulations was a risky business, would not have had a reasonable expectation of success to arrive at the claimed subject-matter upon combination of the teaching in two prior art documents. However, the board pointed out that a reasonable expectation of success does not require certainty. Thus, the board held that in spite of the understandable uncertainties which always characterize experiments using biologic compounds like proteins, the skilled person had no reason to adopt a skeptical attitude. He or she would have had either some expectations of success or, at worst, no particular expectations of any sort, but only a "try and see" attitude, which does not equate with the absence of a reasonable expectation of success (cf. Item 32 of the "Reasons for the Decision" of **T1241/03**). Accordingly, the board denied an inventive step.

(151) The present case is comparable to the case underlying **T1241/03**. However, in the present case the skilled person did not even have to combine the teachings of different prior art documents. Rather, the skilled person could simply rely on his or her common general knowledge that aggregate formation of antibodies increases with increasing concentrations. Accordingly, faced with the problem of providing a stable eculizumab formulation, he or she would have tested a number of standard concentrations and would have found that concentrations lower than 30 mg/ml, e.g. 10 mg/ml, provide for the desired stability. Just like in **T1241/03**, the skilled person had no reason to be skeptical that one of the tested antibody concentrations would solve the problem of providing a stable eculizumab formulation, i.e. a formulation with a monomer content of at least 98% after 36 months of storage at 2-8°C.

(152) We also refute the Proprietor's allegation that a concentration of 10 mg/ml was not typical for pharmaceutical non-IgG1 antibody formulations before the filing date of the opposed patent. To support our view, we refer to the prior art documents **D51** to **D53**, all of which demonstrate that a concentration of 10 mg/ml was also common for non-IgG1 antibodies. **D51** to **D53** were filed in direct response to the Proprietor's allegation that no conclusions for a suitable antibody concentration of non-IgG1 antibodies can be drawn from prior art relating to IgG1 antibodies.

(153) Additionally, it should be considered that the opposed patent does not give any significance of the antibody isotype for the stability of eculizumab formulations. The only mention of isotypes in the opposed patent is found in paragraph [0055] and relates to the recognition of IgG1 and IgG3 antibodies by phagocytic cells. The specific isotype of eculizumab (IgG2/4) is not even mentioned in the opposed patent.

(154) **D51** relates to the pharmaceutical IgG4 antibody natalizumab. The particular focus of **D51** is to provide stable antibody formulations:

> *"Therefore, notwithstanding what has previously been reported in the literature, there exists a need for improved methods of formulating proteins and/or antibodies. There is also a need for stable formulations with large concentrations of antibody or protein, where the activity of the antibody or protein is retained. Also needed are stable formulations of concentrated protein which maintain a fixed volume. Applicants disclose herein stable compositions which can be further utilized to prepare antibody formulations, especially formulations with high concentrations of antibody which do not precipitate out and are stable when stored at the recommended temperatures. The highly concentrated and stable antibody formulations will greatly aid physicians in treating subjects of variable weights."* (p. 1, ll. 25-34 of **D51**)

(155) The concentration of 10 mg/ml is singled out on page 13, lines 2 to 5 of **D51**. Notably, Examples 11 and 12 list all ingredients of two exemplary, stable natalizumab formulations at exactly 10 mg/ml (pp. 23-24 of **D51**). Like the eculizumab composition of the opposed patent, the natalizumab compositions of **D51** are preservative-free. These data show that a concentration of 10 mg/ml is a typical concentration also for non-IgG1 antibody formulations, e.g. for formulations comprising IgG4 antibodies such as natalizumab.

(156) **D52** relates to the IgG4 antibody volociximab (p. 2, ll. 6-8 and p. 8, l. 22 of **D52**). On page 20, lines 19 to 23, **D52** indicates that in a preferred embodiment, the antibody is formulated as a pharmaceutical composition comprising a solution of between about 1.0 and 15.0 mg/ml antibody. **D52** further states that *"in a particularly preferred embodiment the pharmaceutical composition comprises a solution of about*

10.0 mg/mL antibody, […]" (p. 20, ll. 24-26 of **D52**). In a table listing the parameters of a phase II clinical study, the concentration of volociximab compositions for treating patients with metastatic renal cell carcinoma is indicated to be 10 mg/ml (cf. p. 41, "Dosage Form and Strength/Formulation" of **D52**). Hence, also **D52** demonstrates that a concentration of 10 mg/ml is a typical concentration for non-IgG1 antibody formulations.

(157) Finally, **D53** relates to antibodies that regulate the activity of NK cells. Although the preferred antibody is of the IgG4 isotype, reference is also made to IgG1, IgG2 and IgG3 antibodies (cf. claims 15-16 of **D53**). The preferred pharmaceutical composition comprising these antibodies has a concentration of 10 mg/ml:

> *"For example, an antibody present in a pharmaceutical composition of this invention can be supplied at a concentration of 10 mg/mL in either 100 mg (10 mL) or 500 mg (50 mL) single-use vials."* (p. 73, ll. 17-19 of **D53**)

(158) In **D35**, the Proprietor had additionally included information on the isotypes of the listed antibodies. According to **D35**, eculizumab is an IgG2/4 antibody, whereas most of the other antibodies included in the list are of the IgG1 isotype. Firstly, this information is NOT available from the original application; even further, the claim is not delimited to an IgG2/4 antibody. Lastly, IgG2/4 antibodies were known before the filing date of the opposed patent, as evidenced e.g. by **D14**. However, the Proprietor alleged that due to the structural differences between non-IgG1 and IgG1 antibodies, no conclusions for a suitable antibody concentration for eculizumab formulations can be drawn from prior art relating to IgG1 antibodies. Moreover, the Proprietor argued that none of the five non-IgG1 antibodies in **D35** were formulated at a concentration of 10 mg/ml. Based on these observations, the Proprietor alleged that, before the filing date of the opposed patent, a concentration of 10 mg/ml was not typical for non-IgG1 antibodies.

(159) Overall, **D35** as presented by the Proprietor himself in combination with **D51**, **D52** and **D53** show that, before the filing date of the opposed patent, a concentration of 10 mg/ml was not only typical for pharmaceutical formulations of IgG1 antibodies, but also common for non-IgG1 antibodies such as IgG4. In view thereof, the skilled

person would certainly have considered a concentration of 10 mg/ml for the IgG2/4 antibody eculizumab, e.g. when performing stability studies as outlined above.

(160) **D54** also cannot improve the situation of the Proprietor; it is merely a declaration which states more or less that dimer formation is not desirable in an antibody formulation which we do not deny.

(161) It is furthermore respectfully submitted that from all the additional declarations it would appear that none of the experiments provided therein have been carried out before the filing date of the opposed patent. Thus, even if an effect is shown in any of these declarations, this effect was not known at the filing date of the opposed patent. That is, **the alleged invention had not been made at the filing date of the opposed patent** and thus should not be rewarded with a patent.

(162) It seems that

- either it was obvious to provide for a 10 mg/ml formulation concentration for the **D1** antibody eculizumab formulation: then the patent would be obvious and not inventive;
- alternatively, it was particularly surprising that the 10 mg/ml formulation was advantageously stable; then, this alleged invention had not been made at the time of filing and the patent should not be inventive for that reason.

Regarding the additional document **D55**:

(163) **D55** is an even further declaration of Dr. Miano. Again, comparative experiments between 10 mg/ml and 30 mg/ml where carried out.

(164) Even if these data are admitted into the proceedings (we definitely deny that this should be allowed) and even if they are considered to show that a 10 mg/ml eculizumab formulation would have had less aggregation propensity than the 30 mg/ml formulation, it is respectfully submitted that it was well-known in the prior art that antibody formulations with lower antibody concentration had less aggregation propensity, as amply shown above. This is one of the reasons that the 10 mg/ml concentration is the preferred concentration for antibody formulations of commercially available products.

(165) Also, if this was indeed considered as a surprising effect, again, it was not known at the filing date of the opposed patent and thus certainly should not be taken into account for determining inventive step of the opposed patent.

Summary

(166) The Main Request is not inventive in view of the above cited art even if the approach of the Proprietor regarding a re-formulation of the technical problem is taken into account.

2.2.2.3 First Auxiliary Request

(167) The First Auxiliary Request is late filed and should not be admitted into the proceedings; see *supra*. Indeed, it was already considered to be late filed in first instance proceedings. It is respectfully submitted that the Proprietor does not discuss the significance of the amendments for the auxiliary requests, apart from pointing to original disclosure thereof. This particular auxiliary request also seems to be unreasonable in view of the extensive discussion of the Proprietor that SEQ ID NO: 4 would actually NOT be present in the actual eculizumab antibody and this request thus also contravenes Rule 80 EPC.

(168) For the sake of completeness, we will address this request nevertheless.

Auxiliary Request 1 lacks inventive step over **D1** as closest prior art (in combination with the documents as cited above for the Main Request)

(169) While **D1** does not comprise a reference to the exact sequences of SEQ ID NO:2 and SEQ ID NO:4, it does refer to preferred eculizumab antibodies. These references are to be found on page 5, left column last 4 lines to right column, line 8. In particular, **D1** refers to – and incorporates by reference – both **D17** and **D13**. While these documents, as discussed above, do not explicitly point to the above two sequences, there is certainly no advantage associated with these sequences as well. The opposed patent is silent as to any advantage of these sequences. The opposed

patent is also silent as to the subtype-issue which is alleged by the Proprietor to lend an advantage to an antibody characterized by these sequences[6].

(170) **D57** certainly cannot help. Again – this is another declaration which is late filed, had long before been available to the Proprietor and refers to facts which cannot be derived from the original application. It should definitely be disregarded. **D57** explains at length that the commercial eculizumab is a "*unique, non-naturally occurring protein engineered antibody*". While this may be true, this fact is not derivable from the patent. It is also fully unclear how this can have any impact on e.g. the stability of the formulation, in particular in view of the fact that there is no reference to stability whatsoever in the original application. It is further unclear how the Proprietor can state in their item 5.5.7, second sentence in that same context that eculizumab has a heavy chain shown in SEQ ID NO:2 of the patent and the light chain sequence shown in SEQ ID NO:4 of the patent, as this is evidently not true.

(171) Even further, the opposed patent is silent as to whether the eculizumab used in their examples is actually the eculizumab with SEQ ID NO:2 and 4 (apparently, it is not, as the opposed patent refers to Soliris™ in that regard).

(172) In any case – there is no technical effect whatsoever associated with the selection of the above sequences. The Proprietor again attempts to re-define the problem and show an effect by way of adding late-filed information to the original disclosure which, however, simply is insufficient to support these allegations.

(173) The First Auxiliary Request is thus not inventive for the exact same reasons as the Main Request.

2.2.2.4 Second Auxiliary Request

(174) The Second Auxiliary Request differs from the First Auxiliary Request in its different definition of the light chain. The light chain is now defined as ".. consisting of residues 23 to 214 of SEQ ID NO:4".

[6] It must be kept in mind that these sequences are not even the correct ones!

(175) As argued above, this request is late filed. The entire sequence discussion only came up in oral proceedings (initiated by the Proprietor). All requests directed to a definition of sequences and relying on respective arguments are thus clearly late filed. The present request was not even submitted in first instance proceedings.

(176) It is also *prima facie* not allowable, as it contravenes **Article 123(2) EPC**.

(177) Even the Proprietor does not find explicit disclosure of the specifically selected residues in the original application. They attempt to replace this missing disclosure by yet another declaration, **D59**. Our arguments against admitting this document into the proceedings are herewith repeated. Just for the sake of discussion: **D59** actually confirms that the person skilled in the art would not have been able to understand in a clear and unambiguous way that the actual light chain would start at position 23 of SEQ ID NO:4. The expert actually needs seven paragraphs and reference to four further documents (Exhibits B – E) to come to the conclusion on which sequence the light chain would actually have. The argument of the expert pre-supposes that the person skilled in the art would

(1) Recognize the methionine at the begin of the sequence
(2) Recognize that the following amino acids are mostly hydrophobic, and
(3) Recognize that the light chain was longer than the average light chain

and would conclude that SEQ ID NO:4 must necessarily include its signal peptide.

(178) While we do not wish to refute the fact that signal peptides are preceded by a methionine and that they are composed mostly of hydrophobic amino acids, the question in the present case is whether or not the person skilled in the art would have automatically read that information into the original disclosure of SEQ ID NO:4. This is certainly not the case.

(179) First of all, there is no reason for the person skilled in the art to assume that SEQ ID NO:4 is incorrect. This would however be the first pre-requisite according to standard EP practice which would have to be fulfilled for the correction of an error or the assumption of a different implicit disclosure. Indeed, SEQ ID NO:2 is the correct final sequence. The person skilled in the art would not have assumed that one sequence of the antibody did not include the signal peptide while the other sequence did

include the signal peptide. Also, the patent clearly states that the final antibody comprises both SEQ ID NO:2 and 4. Thus, the immediate assumption would be that both sequences are sequences without signal peptide.

(180) As regards the above three points in detail:

1) While methionine is the usual starting point for signal peptides, it is also a "normal" amino acid which occurs at other points of a peptide. See e.g. position 26 of SEQ ID NO:4.
2) Hydrophobic amino acids also occur throughout a peptide; e.g. positions 24 and 26 are also hydrophobic amino acids.
3) Having a more than average length just means that the peptide is longer than average, which is in and out of itself not surprising at all[7].

(181) Thus, the person skilled in the art would not have been aware of an error in SEQ ID NO:4 and would also not have been able to supplement the correct information.

(182) To arrive at this correct information, the Proprietor actually makes a leap in the argumentation. This leap is to select the "human kappa light chain" information in their cited Kabat reference. The opposed patent, however, does not disclose that the eculizumab antibody in question comprises a kappa light chain. Therefore, the skilled person would not have known to look specifically for information on the kappa light chain. The entire further arguments of the Proprietor are therefore flawed.

(183) The Proprietor then makes a further leap to "Human Kappa Light Chains Subgroup I". There is no explanation why the person skilled in the art would have turned to Subgroup I. Even if this second leap was to be followed, the Proprietor then needs to make an even further assumption, namely that the signal peptide has a length of 22 amino acids. It is entirely unclear to the Respondent where this information should come from. Certainly, it is not disclosed in the patent. Even further, it is immediately evident from **Exhibit D of D59** that there are quite a few different signal peptide lengths, even within the selected subgroup I of the kappa light chain. Even if it were true that the "vast majority" of human kappa light chains of subgroup I then started

[7] As a further note – while the Proprietor refers to a document supporting the allegation of average length, they have not included it and this allegation is thus totally unsubstantiated

with the sequence DIQM, this is by no means the necessary direct and unambiguous disclosure which would allow an Applicant or Proprietor to replace an erroneous description in the original application.

(184) In direct reaction to this new argument of the Proprietor and in case the newly filed documents of the Proprietor should be admitted into the proceedings, we herewith refer to document **D62**, enclosed herewith. This document is the Drug Bank information on the monoclonal antibody Denosumab®, which is a human IgG2 monoclonal antibody with a kappa light chain of subgroup 1. The light chain sequence starts with the amino acids EIVL (and thus not DIQM). Please see on the first page, "Sequences", second paragraph of **D62**. Similarly, please see **D63**, which is the DrugBank information on Ranibizumab®. Ranibizumab® is an IgG1 kappa immunoglobulin of subgroup I; again, the light chain does not start with DIQM but with DIQL. **D64** is a US patent which describes anti-IgGE antibodies. **D64**, in column 37, example 4 and Table 8 shows that the antibody of interest is an antibody with a V_L region kappa subgroup I. and comprises a light chain which starts with DIQL (see SEQ ID NO:9 which is the light chain of the V.1 version antibody "mae11" described in table 8)[8].

(185) This clearly contradicts the allegation of the Proprietor that the vast majority of human kappa light chains of subgroup I start with the sequence DIQM.

(186) As there is no unambiguous disclosure for a signal peptide and even less unambiguous disclosure for a signal peptide with a length of 22 amino acids, **this request is not allowable under Article 123(2) EPC.**

(187) <u>Just as one further remark:</u> should the BoA follow the interpretation of the Proprietor regarding the term "eculizumab" for the Main Request (where the Proprietor argues that the skilled person would read "eculizumab" to be delimited to an antibody consisting of SEQ ID NO:2 and 4), then the present request would

[8] As an aside: the antibody is also a commercial antibody, which is marketed under the name omalizumab®.

contravene Article 123(**3**) EPC as well. This is because the present request allows for a different antibody (i.e. a shorter sequence) than the Main Request, following the interpretation of the Proprietor.

(188) The present request also contravenes Article 56 EPC for the same reasons as the Main Request and the First Auxiliary Request.

2.2.2.5 Third Auxiliary Request

(189) This request was rejected as being late filed in first instance proceedings and should thus also not be admitted now. It is also not convergent with the higher ranking requests.

(190) This request includes the feature that the pH of the composition is 7.0. The request is not allowable for the additional reason that it is *prima facie* not formally allowable. The Proprietor refers to [0090] of the original application for disclosure. This disclosure, however, refers to other antibody formulations. As can be taken from **D56** (which are the abstracts of the documents cited in the passage), they are not concerned with eculizumab. The person skilled in the art would not have combined the upper limit of the range of 5.0 to 7.0 cited for these different formulations with the very specific eculizumab formulation of claim 1. Proprietor's earlier submission dated November 21, 2018 is recalled where they state in their item 3.10, last three sentences that "*…each antibody needs to be considered individually. This requires a uniquely-designed, detailed research program to determine the ideal composition for a given antibody. A person skilled int eh art would not have had any confidence that applying a formulation found to be successful for a different antibody would also be successful with eculizumab*."

(191) The Third Auxiliary Request thus contravenes Article 123(2) EPC.

(192) It is respectfully referred to [0013] of **D22** which discloses a pH of 6 - 8. Similarly, **D20** lists in Table 1 formulations which are in that same range, many of them being at pH 7. Indeed, it is advisable and well known to use a pH around 7.0 for intravenous infusion; see in particular those antibodies which are to be administered via IV infusion and which all are at or come close to pH 7.

(193) Apart from the above, this feature is also not associated with any technical effect; even the Proprietor does not provide any substantiated arguments in favor of a pH of 7 in the justification of their providing this auxiliary request at all[9].

(194) The Third Auxiliary Request is thus also not inventive, for essentially the same reasons as cited for the earlier auxiliary requests.

(195) The same considerations apply to the **Fourth and Fifth Auxiliary Requests** which are merely combinations of earlier requests, which are also not substantiated whatsoever as to their relevance by the Proprietor.

2.2.2.6 Sixth Auxiliary Request

(196) This request is again divergent from the earlier requests and has not been substantiated at all by the Proprietor as to its relevance. It includes the feature of intravenous administration. As **D1** in its preferred embodiment related to eculizumab (see [0082]) also discloses intravenous administration, the same considerations as for the Main Request apply here as well.

2.2.2.7 Auxiliary Request 7

(197) This request combines AR 3 and 6, again without any explanation of the Proprietor as to its significance. This makes is exceedingly difficult for the Respondent to react

[9] Please note – should **D55** be considered, it shows in its Figures 1 and 2 that pH 6.5 was actually better than pH 7.0!

appropriately. Should new arguments be made in the further proceedings regarding the relevance of all these auxiliary requests, we already preemptively state that these arguments would be clearly too late.

(198) In any case, what can be said already now is that this request suffers from the same issues as Auxiliary Requests 3 and 6 and is thus on the one hand not admissible and on the other hand not patentable.

2.2.2.8 Auxiliary Request 8

(199) Apart from the above discussed late-filing issues and lack of substantiation, this request is also again diverging from the earlier requests.

(200) It includes the feature of Auxiliary Request 7 (intravenous infusion) which is known from **D1**, and the further feature that the composition is diluted to a specific final concentration prior to administration. The disclosure has been taken from the general part of the specification and the opposed patent comprises no indication why this specific dilution could be useful or particularly advantageous. Indeed, the paragraph from which this feature has been taken deals with generally known routes for administration and thus already clarifies that this feature has not been associated with any particular surprise.

(201) The Proprietor did not address the significance of this request in the Grounds of appeal. Similarly, they did not address the significance of this request in their original submission of November 21, 2018, where they introduced this request. Thus, the Respondent is at a loss which possible arguments might support an inventive step of

this feature. We reserve the right to file additional arguments or documents in case the Proprietor comes up with further arguments in that regard.

(202) **In sum – none of the auxiliary requests is patentable.**

(203) The Decision revoking the patent is correct. The opposed patent fails to meet the requirements of the EPC. The above-mentioned facts and those presented in previous submissions in the opposition proceedings fully justify the request to revoke the opposed patent in its entirety.



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Encl.:

D1 – US 2005/0191298 A1
D3 – Hillmen et al., (2004) N. Engl. J. Med. 350(6): 552 – 559
D13 – Thomas et al., (1996), Mol. Immunol. 33(17/18): 1389-1401
D14 – Mueller et al, (1997), Mol. Immunol. 34 (6) :441 - 452
D17 – US 6,355,245 B1
D19 – EMEA, CPMP, Note for Guidance, 20 February 2003
D20 – Wang et al., (2007) J. Pharm. Sci. 96(1): 1 – 26 (January 2007)
D21 – Daugherty et al., (2006), Adv. Drug. Del. Rev.58: 686 - 706
D22 – US 2005/027166 A1
D29 – US 6,171,586 B1
D30 – Maloney et al., (1997), Blood 90(6), 2188 - 2195

D31 –Commercial monoclonal antibodies 2006 (all modes of administration)
D32 – Commercial monoclonal antibodies 2006 (IV administration)
D35 – Summary of antibodies listed in D20/D21 Tables
D46 – Emery et al., (2006), Arthritis and Rheumatism, Vol 54(5), 1390 - 1400
D50 – ICH Guidelines
D51 – WO 2004/071439
D52 – WO 2005/092073
D53 – WO 2006/003179
D61 – T 386/89
D62 – Denosumab, Drug Bank, accessed November 04, 2019
D63 – Ranibizumab, Drug Bank, accessed November 05, 2019
D64 – US 6,329,509 B1

Ranibizumab

Targets (1)

IDENTIFICATION

Name	Ranibizumab
Accession Number	DB01270
Type	Biotech
Groups	Approved
Biologic Classification	Protein Based Therapies **Monoclonal antibody (mAb)**
Description	Ranibizumab is a recombinant humanized IgG1 kappa isotype monoclonal antibody fragment designed for intraocular use. Ranibizumab binds to and inhibits the biologic activity of human vascular endothelial growth factor A (VEGF-A). Ranibizumab has a molecular weight of approximately 48 kilodaltons and is produced by an E. coli expression system in a nutrient medium containing the antibiotic tetracycline (tetracycline is not detectable in the final product). Ranibizumab is marketed under the name Lucentis®. It is indicated for the treatment of macular edema after retinal vein occlusion, age-related macular degeneration (wet), and diabetic macular edema.
Protein structure	
Protein chemical formula	$C_{2158}H_{3282}N_{562}O_{681}S_{12}$
Protein average weight	48349.611 Da
Sequences	>Ranibizumab Light Chain DIQLTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIYFTSSLHSGVPS RFSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLT LSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC >Ranibizumab Heavy Chain EVQLVESGGGLVQPGGSLRLSCAASGYDFTHYGMNWVRQAPGKGLEWVGWINTYTGEPTY AADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYPYYYGTSHWYFDVWGQGTLVT VSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHL Download FASTA Format
Synonyms	rhuFab V2
External IDs	RG-3645

Prescription Products

Show 10 entries Search

NAME	DOSAGE	STRENGTH	ROUTE	LABELLER	MARKETING START	MARKETING END
Lucentis	Solution	10 mg	Intravitreal	Novartis	2014-09-18	Not applicable
Lucentis	Injection	10 mg/ml	Intravitreal	Novartis	2007-01-22	Not applicable

Indication For the treatment of patients with macular edema after retinal vein occlusion, age-related macular degeneration (wet), and diabetic macular edema.

Associated Conditions

Diabetic Macular Edema (DME)

Macular Edema (ME)

Retinopathy, Diabetic

Subfoveal Choroidal Neovascularization

Wet Age-Related Macular Degeneration

Pharmacodynamics Ranibizumab is a recombinant humanized IgG1 kappa isotype monoclonal antibody fragment designed for intraocular use. Ranibizumab is a VEGF-A antagonist that binds to and inhibits the biologic activity of active forms of human VEGF-A, including the cleaved form ($VEGF_{110}$). VEGF-A has been shown to cause neovascularization (angiogenesis) and an increase in vascular permeability, which is thought to contribute to the progression of the neovascular form of age-related macular degeneration (AMD).

Mechanism of action Ranibizumab binds to the receptor binding site of active forms of VEGF-A, including the biologically active, cleaved form of this molecule, $VEGF_{110}$. The binding of ranibizumab to VEGF-A prevents the interaction of VEGF-A with its receptors (VEGFR1 and VEGFR2) on the surface of endothelial cells, reducing endothelial cell proliferation, vascular leakage, and new blood vessel formation.

TARGET	ACTIONS	ORGANISM
(A) Vascular endothelial growth factor A	Not Available	Humans

ADDITIONAL DATA AVAILABLE

Adverse Effects

Comprehensive structured data on known drug adverse effects with statistical prevalence. MedDRA and ICD10 ids are provided for adverse effect conditions and symptoms.

LEARN MORE

ADDITIONAL DATA AVAILABLE

Contraindications

Structured data covering drug contraindications. Each contraindication describes a scenario in which the drug is not to be used. Includes restrictions on co-administration, contraindicated populations, and more.

LEARN MORE

ADDITIONAL DATA AVAILABLE

Blackbox Warnings

Structured data representing warnings from the black box section of drug labels. These warnings cover important and dangerous risks, contraindications, or adverse effects.

LEARN MORE

Absorption After monthly intravitreal injections, maximum serum concentrations are minimal around 0.3 ng/mL to 2.36 ng/mL.

Volume of distribution Volume of distribution is insignificant.

Protein binding Plasma protein binding is insignificant.

Metabolism Metabolism was not quantified.

Route of elimination Not Available

Half life Approximately 9 days.

Clearance Clearance was not quantified.

Toxicity The most common toxic effects to the eye are eye pain, vitreous floaters, increased intraocular pressure, conjunctival hemorrhage, intraocular inflammation, and foreign body sensation. Also arterial thromboembolic events have occurred in patients.

Affected organisms Humans and other mammals

INTERACTIONS

Drugs

Drug Interactions

This information should not be interpreted without the help of a healthcare provider. If you believe you are experiencing an interaction, contact a healthcare provider immediately. The absence of an interaction does not necessarily mean no interactions exist.

ALL DRUGS | APPROVED | VET APPROVED | NUTRACEUTICAL | ILLICIT | WITHDRAWN | INVESTIGATIONAL | EXPERIMENTAL

Show 10 entries Search

DRUG	INTERACTION
Abciximab	The risk or severity of adverse effects can be increased when Abciximab is combined with Ranibizumab.
Abituzumab	The risk or severity of adverse effects can be increased when Ranibizumab is combined with Abituzumab.
Abrilumab	The risk or severity of adverse effects can be increased when Ranibizumab is combined with Abrilumab.
Adalimumab	The risk or severity of adverse effects can be increased when Adalimumab is combined with Ranibizumab.
Adecatumumab	The risk or severity of adverse effects can be increased when Ranibizumab is combined with Adecatumumab.
Aducanumab	The risk or severity of adverse effects can be increased when Ranibizumab is combined with Aducanumab.
Afelimomab	The risk or severity of adverse effects can be increased when Ranibizumab is combined with Afelimomab.
Alemtuzumab	The risk or severity of adverse effects can be increased when Alemtuzumab is combined with Ranibizumab.
Alendronic acid	The risk or severity of jaw osteonecrosis and anti-angiogenesis can be increased when Ranibizumab is combined with Alendronic acid.
Alirocumab	The risk or severity of adverse effects can be increased when Ranibizumab is combined with Alirocumab.

ADDITIONAL DATA AVAILABLE

Showing 1 to 10 of 336 entries < 1 2 3 4 5 ... 34 >

Food Interactions

No food effects found.

REFERENCES

General References

1. Gaudreault J, Fei D, Beyer JC, Ryan A, Rangell L, Shiu V, Damico LA: Pharmacokinetics and retinal distribution of ranibizumab, a humanized antibody fragment directed against VEGF-A, following intravitreal administration in rabbits. Retina. 2007 Nov-Dec;27(9):1260-6. [PubMed:18046235]
2. Gaudreault J, Fei D, Rusit J, Suboc P, Shiu V: Preclinical pharmacokinetics of Ranibizumab (rhuFabV2) after a single intravitreal administration. Invest Ophthalmol Vis Sci. 2005 Feb;46(2):726-33. [PubMed:15671306]
3. Bakri SJ, Snyder MR, Reid JM, Pulido JS, Ezzat MK, Singh RJ: Pharmacokinetics of intravitreal ranibizumab (Lucentis). Ophthalmology. 2007 Dec;114(12):2179-82. [PubMed:18054637]
4. Kourlas H, Abrams P: Ranibizumab for the treatment of neovascular age-related macular degeneration: a review. Clin Ther. 2007 Sep;29(9):1850-61. [PubMed:18035167]

External Links

KEGG Drug	D05697
PubChem Substance	46506960
ChEMBL	CHEMBL1201825
Therapeutic Targets Database	DAP001260
PharmGKB	PA164746012
RxList	RxList Drug Page
Drugs.com	Drugs.com Drug Page
Wikipedia	Ranibizumab

CLINICAL TRIALS

Drugs

Clinical Trials Show 10 entries Search

PHASE	STATUS	PURPOSE	CONDITIONS	COUNT
0	Completed	Treatment	Age-Related Macular Degeneration (ARMD)	1
0	Completed	Treatment	Cutaneous Neurofibromas / Neurofibromatosis Type 1	1
0	Completed	Treatment	Retinopathy, Diabetic	1
1	Completed	Treatment	Age Related Macular Degeneration (AMD)	1
1	Completed	Treatment	Age-Related Macular Degeneration (ARMD)	1
1	Completed	Treatment	Central Retinal Vein Occlusion (CRVO)	2
1	Completed	Treatment	Central Serous Chorioretinopathy (CSC)	1
1	Completed	Treatment	Choroidal Melanoma	1
1	Completed	Treatment	Choroidal Melanoma / Radiation Retinopathy	1
1	Completed	Treatment	Conjunctival Neoplasms	1

Showing 1 to 10 of 359 entries < 1 2 3 4 5 ... 36 >

PHARMACOECONOMICS

Manufacturers Not Available

Packagers Genentech Inc. Novartis AG

Dosage forms Show 10 entries Search

FORM	ROUTE	STRENGTH
Injection, solution	Intravitreal	10 mg/1mL
Injection, solution	Intravitreal	10 mg/ml
Injection, solution	Intravitreal	6 mg/1mL
Solution	Intravitreal	10 mg

Showing 1 to 4 of 4 entries < 1 >

Prices Show 10 entries Search

UNIT DESCRIPTION	COST	UNIT
Lucentis 0.5 mg vial	2340.0USD	vial

Showing 1 to 1 of 1 entries < 1 >

DrugBank does not sell nor buy drugs. Pricing information is supplied for informational purposes only.

Patents Show 10 entries Search

PATENT NUMBER	PEDIATRIC EXTENSION	APPROVED	EXPIRES (ESTIMATED)
CA2286230	No	2008-06-10	2018-04-03

Showing 1 to 1 of 1 entries < 1 >

PROPERTIES

State Liquid

Experimental Properties

PROPERTY	VALUE	SOURCE
melting point (°C)	Not applicable	From MSDS.
boiling point (°C)	Approximately 100 degrees C	From MSDS.
water solubility	Soluble in water.	From MSDS.

TAXONOMY

Drugs

Description	Not Available
Kingdom	Organic Compounds
Super Class	Organic Acids
Class	Carboxylic Acids and Derivatives
Sub Class	Amino Acids, Peptides, and Analogues
Direct Parent	Peptides
Alternative Parents	Not Available
Substituents	Not Available
Molecular Framework	Not Available
External Descriptors	Not Available

TARGETS

1. Vascular endothelial growth factor A

Details

Kind	Protein	**General Function**	Vascular endothelial growth factor receptor binding
Organism	Humans	**Specific Function**	Growth factor active in angiogenesis, vasculogenesis and endothelial cell growth. Induces endothelial cell proliferation, promotes cell migration, inhibits apoptosis and induces permeabilization of...
Pharmacological action	Yes	**Gene Name**	VEGFA
		Uniprot ID	P15692
		Uniprot Name	Vascular endothelial growth factor A
		Molecular Weight	27042.205 Da

References

1. Gaudreault J, Fei D, Rusit J, Suboc P, Shiu V: Preclinical pharmacokinetics of Ranibizumab (rhuFabV2) after a single intravitreal administration. Invest Ophthalmol Vis Sci. 2005 Feb;46(2):726-33. [PubMed:15671318]
2. Michels S, Rosenfeld PJ: [Treatment of neovascular age-related macular degeneration with Ranibizumab/Lucentis]. Klin Monbl Augenheilkd. 2005 Jun;222(6):480-4. [PubMed:15977026]
3. Rosenfeld PJ, Rich RM, Lalwani GA: Ranibizumab: Phase III clinical trial results. Ophthalmol Clin North Am. 2006 Sep;19(3):361-72. [PubMed:16935211]
4. Nguyen QD, Tatlipinar S, Shah SM, Haller JA, Quinlan E, Sung J, Zimmer-Galler I, Do DV, Campochiaro PA: Vascular endothelial growth factor is a critical stimulus for diabetic macular edema. Am J Ophthalmol. 2006 Dec;142(6):961-9. Epub 2006 Aug 2. [PubMed:16876491]
5. Pieramici DJ, Avery RL: Ranibizumab: treatment in patients with neovascular age-related macular degeneration. Expert Opin Biol Ther. 2006 Nov;6(11):1237-45. [PubMed:17049026]
6. Gaudreault J, Fei D, Beyer JC, Ryan A, Rangell L, Shiu V, Damico LA: Pharmacokinetics and retinal distribution of ranibizumab, a humanized antibody fragment directed against VEGF-A, following intravitreal administration in rabbits. Retina. 2007 Nov-Dec;27(9):1260-6. [PubMed:18046235]
7. Ferrara N, Damico L, Shams N, Lowman H, Kim R: Development of ranibizumab, an anti-vascular endothelial growth factor antigen binding fragment, as therapy for neovascular age-related macular degeneration. Retina. 2006 Oct;26(8):859-70. [PubMed:17091864]
8. Bakri SJ, Snyder MR, Reid JM, Pulido JS, Ezzat MK, Singh RJ: Pharmacokinetics of intravitreal ranibizumab (Lucentis). Ophthalmology. 2007 Dec;114(12):2179-82. [PubMed:18054632]
9. Kourlas H, Abrams P: Ranibizumab for the treatment of neovascular age-related macular degeneration: a review. Clin Ther. 2007 Sep;29(9):1850-61. [PubMed:18035167]
10. Spitzer MS, Ziemssen F, Bartz-Schmidt KU, Gelisken F, Szurman P: Treatment of age-related macular degeneration: focus on ranibizumab. Clin Ophthalmol. 2008 Mar;2(1):1-14. [PubMed:19668384]

Drug created on May 16, 2007 14:14 / Updated on November 04, 2019 15:42

This project is supported by the **Canadian Institutes of Health Research** (award #111062), **Alberta Innovates - Health Solutions**, and by **The Metabolomics Innovation Centre (TMIC)**, a nationally-funded research and core facility that supports a wide range of cutting-edge metabolomic studies. TMIC is funded by **Genome Alberta**, **Genome British Columbia**, and **Genome Canada**, a not-for-profit organization that is leading Canada's national genomics strategy with funding from the federal government. Maintenance, support, and commercial licensing is provided by **OMx Personal Health Analytics, Inc.** Designed by **Educe Design & Innovation Inc.**

LIVERPOOL HOSPITAL

NSW GOVERNMENT Health
South Western Sydney Local Health District

Drug Guideline Title: Salbutamol

Summary: Salbutamol is a bronchodilator, Beta $(\beta)_2$ adrenoreceptor stimulant, with some β_1 effects at high dosage levels.

Approved by: ICU Director

Publication (Issue) Date: June 2014

Next Review Date: June 2017

Replaces Existing Drug Guideline: Salbutamol

Previous Review Dates: 2002, 2004

1. Introduction:

The risk addressed by this policy:

Patient Safety

The Aims / Expected Outcome of this policy:

Salbutamol will be administered safely and appropriately without any adverse side effects.

Related Standards or Legislation

NSQHS Standard 1 Governance

National Standard 4 Medication Safety

Related Policies

- LH_PD2013_C03.01 Drug Administration
- LH_PD2010_C03.00 Drug Prescribing
- LH_PD2008_C03.12 Administration of IV Medication

2. Policy Statement:

- All care provided within Liverpool Hospital will be in accordance with infection control, manual handling and minimisation and management of aggression guidelines.
- Medications are to be prescribed and signed by a medical officer/authorised nurse practitioner (NP) unless required during an emergency.
- All drugs administered during an emergency (under the direction of a medical officer/authorised nurse practitioner) are to be documented during the event, then prescribed and signed following the event.

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- Medications are to be given at the time prescribed (as close to the time as is possible when multiple drugs require 'same time' administration and, when the nurse is caring for more than one patient, recognition is given to a possible short delay to administration – antibiotics and other lifesaving drugs are to be prioritised) and are to be signed by the administering nurse.
- Parenteral medication prescriptions and the drug are to be checked with a second registered or endorsed enrolled nurse prior to administration. The "rights of drug administration" must be followed: right: patient, drug, dose, route, administration, time, reason for the drug, documentation, education and evaluation/outcome.
- Adverse drug reactions are to be documented and reported to a medical officer.
- Medication errors are to be reported using the hospital electronic reporting system: IIMS.
- Guidelines are for adult patients unless otherwise stated

3. Principles / Guidelines

Actions[1,2,3]

- Salbutamol is a bronchodilator, Beta (β) -2 adrenoreceptor stimulant, with some β -1 effects at high dosage levels.
- β - 1 stimulation causes an increase in heart rate, contractility and excitability.
- β - 2 stimulation causes bronchodilatation and vasodilatation.
- It causes uterine smooth muscle relaxation and is used to help prevent premature labor.

Indications[1,2,3]

- Relief of severe bronchospasm
- Relief of bronchospam in asthma.
- Any other cause of bronchospasm, such as:
  - Anaphylaxis
  - Smoke inhalation
  - Pulmonary oedema
  - Pneumonia
  - Bronchitis
- Shortness of breath with expiratory wheeze
- Inhibition of threatened preterm labor between 24-30 weeks. It is used as a tocolytic agent. The aim of tocolysis is to delay preterm birth to allow time for maternal administration of corticosteroids and in-utero transfer to a tertiary perinatal centre, thereby reducing neonatal morbidity and mortality.

Note that there is little evidence to support the use of IV Salbutamol in adults and generally continuously nebulised Salbutamol is more effective

Contraindications[1,2,3]

- Hypersensitivity.
- Severe pre-eclampsia
- Uterine bleeding
- Premature membrane rupture with chorioamnionitis

Precautions[1,2,3]

- During intravenous infusion of Salbutamol Injection for premature labour, careful monitoring of maternal pulse rate and blood pressure is recommended in addition to careful observation of foetal heart rate and status of the infant.
- It can induce metabolic changes such as hypokalaemia and increased blood glucose levels. Monitor and replace potassium, patient may require an insulin infusion.
- Monitor for tachycardia, cardiac dysarrhythmias, flushing and headache.

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Significant Interactions[4]
- When corticosteroids are used concurrently it may exacerbate hyperglycemia.
- Should be given only with extreme caution to patients who have already received large doses of sympathomimetics.

Adverse Effects[1,2,3,4]
- Increase in heart rate. This response is dose dependent, but is rarely a major problem.
- Hypotension may occur.
- By affecting pulmonary mismatch, can actually cause increased hypoxia ("paradoxical hypoxia"). Hence pulse oximetry and supplemental oxygen should be used.
- Fine tremor: Due to a direct action on skeletal muscle. Occurs in 20% of patients.
- Hand tremors, nervousness and restlessness. Less common reactions such as nausea, vomiting and dizziness have been reported.
- Slight stinging / pain or a sensation of warmth may occur after injection
- Hypokalaemia

Presentation[1]
- **Nebulised Solution:** Salbutamol Nebules 5mg in 2.5ml
- **Intravenous Solution**
  - 500 mcg /1 ml ampoule
  - 5 mg / 5 ml ampoule (obstetric)

Administration Guidelines[2,3,4,5]
Nebuliser Administration Guidelines:
- Give 5mg (2.5ml) salbutamol via a nebuliser.
- This dose may be repeated whenever necessary.
- If the patient is in extremis, give nebulised salbutamol **continuously**.

Continuous nebulised salbutamol.
NB: intravenous salbutamol preparation is not to be used for nebulisation. Nebulised solution must be clearly labeled and administered via a syringe driver connected to the nebuliser
Preparation and Administration: **For 5mg/hr**, draw up nebulised salbutamol solution 5mg in 2.5ml in a 50ml syringe (2.5ml x 20 nebuliser ampoules = 50ml in total; 100mg in 50ml = 2mg/ml)
Infuse @ a rate of 2.5ml/hr (5mg/hr) via syringe driver that is connected to the nebuliser bowl. For continuous infusion use the continuous nebuliser kit (Neb. Plus RF_1 nebuliser valve kit).

Intravenous Administration Guidelines

Intravenous Dose for Respiratory: Bronchospasm / Asthma[1,3]
Bolus Loading Dose:
- Give 500 micrograms Salbutamol as a slow IV bolus over 2 to 10 minutes.
- May be repeated if necessary, then commence infusion.

Infusion:
- Dilute 5mg/5mL Salbutamol (obstetric) with 45ml sterile 0.9% sodium chloride, to give a final concentration of 5mg /50ml =100 micrograms/mL.
- Commence at 3mL/hr (5 micrograms/min) and titrate up to 12mL/hr (20 micrograms/min), depending on the patient response.

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- Nebulised Salbutamol is administered concurrently (If inhaled therapy is not feasible or if there is no response to nebulised therapy, intravenous salbutamol can be considered in a highly monitored situation; however, there is no evidence that it is better than inhaled salbutamol)
- Weaning the Salbutamol infusion should not be contemplated until the patient has been stable for at least 24 hours, and is extubated.
- If adrenaline is also being administered, this should be weaned first.
- Decrease the Salbutamol infusion slowly whilst observing the patient closely for recurrent bronchospasm.
- Nebulised Salbutamol is administered concurrently.

Threatened Preterm Labor – Inhibition between 24 and 33 weeks[7]:

- Dilute 5mg/5mL Salbutamol (obstetric) to 45mL sterile 0.9% normal saline, to give a final concentration of 100 micrograms/mL.
- Administer 6-30mL/hour and adjust to obtain desired response.
- Starting dose is at 6mL /hr and increase the rate by 3ml /hr every 10 minutes until there is a suitable response, either cessation of contractions or a reduction in the frequency and strength of contractions.
- Do not exceed 30ml/hr. The maximum dose is determined by the individual's response and may be much less than this in some cases.

Note: For more details on administration of salbutamol in threatened preterm labour please refer to the NSW Health Policy PD2011_025 Maternity - Tocolytic Agents for Threatened Preterm Labour Before 34 Weeks Gestation, http://www0.health.nsw.gov.au/policies/pd/2011/pdf/PD2011_025.pdf

Clinical Considerations[2,3,4,5]

- Nebulised Salbutamol is administered concurrently with intravenous salbutamol infusion.
- When using continous salbutamol infusion via syringe driver – ensure that it is connected to the nebuliser bowl and that it is clearly labeled.
- Salbutamol can cause hypokalaemia – serum potassium concentration should be closely monitored.
- Monitor potassium levels, especially in patients with acute, severe asthma, and when they are receiving drug therapies such as steroids and diuretics.
- Steroids may be administered concurrently, 2nd hourly monitoring of blood glucose levels should be attended.
- Monitor patient for tachycardia and cardiac dysarrhythmias.
- Liaise with obstetrics and maternity staff, when salbutamol is used for prevention of premature labor.
- Monitor maternal and foetal heart rate.

4. Performance Measures

All incidents are documented using the hospital electronic reporting system: IIMS and managed appropriately by the NUM and staff as directed.

5. References / Links

1. MIMS Online, CIAP: NSW Health Department, Copyright MIMS Australia Pty Ltd. February 2012. http://www.use.hcn.com.au
2. The Society of Hospital Pharmacists of Australia 2007, The Australian Injectable Drugs Handbook, 4th Edition, SHPA publications, Australia.
3. eTherapeutic Guidelines: CIAP 2012, http://www.tg.org.au/
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Samsung Bioepis Exhibit No. 1002
Page 13984

Author: CNC – ICU (S. Shunker);
Reviewers: ICU – CNC, CNE, NM, NUM, Staff Specialists, CNS 's, Medical Director, Pharmacist
Endorsed by: A/ Proff M. Parr, Director ICU.

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J A • K E M P

24 June 2019

Our Ref: C411599EP PXT/ALS

STATEMENT OF GROUNDS OF APPEAL ON BEHALF OF ALEXION PHARMACEUTICALS, INC.

Appeal No:	T1087/19-3.3.01
Patent No:	2359834
Application No:	11001632.6
Appellant (Patentee Proprietor):	Alexion Pharmaceuticals, Inc. 100 College Street New Haven Connecticut 06510 United States of America

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2 DOCUMENTS 1

3 MAIN REQUEST: SUMMARY 1

4 SUFFICIENCY OF DISCLOSURE 2

5 INVENTIVE STEP 3

6 AUXILIARY REQUESTS 19

7 SUMMARY 20

1 Requests

1.1 We request that the Opposition Division Decision dated 13 February 2019 is set aside and the patent is maintained as granted.

1.2 If the Board of Appeal is not able to grant a patent on the basis of the above Main Request, we request that the patent be maintained on the basis of one of Auxiliary Requests 1 to 8 discussed below.

1.3 Finally, we request Oral Proceedings in case the Board of Appeal considers any decision other than maintenance of the patent as granted. If Oral Proceedings take place, we will speak in English and request translation into English in the event that any other party intends to speak in a language other than English.

1.4 We request that the content of the Patentee's submissions to the Opposition Division and the documents cited therein are taken into account in the appeal proceedings and form part of the appellant/Patentee's case with respect to Article 12 of the rules of procedure of the Boards of Appeal. In particular, we refer to our submissions of 22 March 2018 and documents D33 to D38 cited therein, our submission of 21 November 2018 and documents D39 to D41 cited therein and our submission of 14 January 2019 and document D54 which was cited therein. These submissions and the cited documents referred to have already been filed and so further copies are not annexed to the present Grounds. We would be happy to file further copies if directed to do so by the Board.

2 Documents

2.1.1 D55 Third declaration of Dr Dino Miano

D56 Abstracts of cross-referenced applications referred to at page 24 of the application as filed

D57 Declaration of Dr Leonard Bell

D58 Declaration of Dr Laural Boone

D59 Declaration of Dr Bruce Andrien

D60 Salbutamol Drug Guidelines

3 Main Request: Summary

3.1 The Patentee's main request is that the Opposition be dismissed and the patent be maintained as granted. In that regard, the Patentee agrees with the comments of the Opposition Division in relation to extension of subject matter under Article 123(2)EPC and Article 76(1) EPC. The Patentee also agrees with the Opposition Division's conclusion with regard to sufficiency, although for clarity, further comments are set out below which summarise the Patentee's position with regard to sufficiency.

Finally, the Patentee requests that the Opposition Division's decision in relation to inventive step is set aside.

4 **Sufficiency of Disclosure**

4.1 The Opponents' arguments in relation to sufficiency before the Opposition Division fell into two separate categories. Firstly, the Opponents argued that the patent specification does not enable the skilled person to produce eculizumab since the light chain sequence provided in the patent (SEQ ID NO: 4) comprises 22 additional amino acids. Secondly, the Opponents argued that claim lacks sufficiency with regard to the formulation of eculizumab.

4.2 In relation to the first point, the Patentee agrees with the Opposition Division's findings. Specifically, the patent provides the full heavy and light chain sequences of eculizumab (SEQ ID NOs: 2 and 4 - see paragraph [0104] of the patent). Although SEQ ID NO: 4 in the patent is the immature light chain sequence of eculizumab and thus contains the signal peptide, the signal peptide would be cleaved off during antibody production in the cell. It would have been well known to the skilled person, as part of his common general knowledge at the effective filing date (see, for example, D41 and D59), that the signal peptide would be cleaved. Therefore, based on the disclosure of the patent and his common general knowledge, the skilled person would have been able to produce eculizumab.

4.3 In relation to the formulation of eculizumab, the Opponents argued that the patent specification does not provide sufficient information to enable a skilled person to produce a formulation of eculizumab which is useful in the treatment of PNH. The Patentee agrees with the Opposition Division that this objection is unfounded.

4.4 It is useful here to briefly set out the legal position in relation to sufficiency of medical use claims. It is well established before the Boards of Appeal that in order to fulfil Article 83 in relation to a medical use claim, (a) the skilled person must be able to produce the composition defined in the claim, and (b) it must be plausible that the composition could be used in the specified treatment. The level of disclosure required in relation to the medical use is set out by Decision T609/02. This describes the now well established principle that the test is a threshold test: all that is required is basic information, either in the patent specification or from the skilled person's common knowledge at the filing date of the patent, which makes the claimed therapeutic effect plausible.

4.5 Applying these principles to the present case, in order to fulfil Article 83, it is necessary that (a) the skilled person could produce a composition comprising 30ml of a 10mg eculizumab per ml sterile preservative free solution, and (b) there must be some indication to make it plausible that the composition would be useful in the treatment of PNH.

4.6 As discussed above, the Opposition Division found, for reasons with which we agree, that the skilled person would be able to produce eculizumab itself. Moreover, the skilled person would have no difficulty in formulating eculizumab into a composition having a 10 mg/ml concentration. The skilled person in the art would be familiar with producing compositions comprising antibodies. Further, there is additional information and guidance provided in the specification at paragraph [0063] onwards. Accordingly, there is no undue burden involved in producing a 10 mg/ml solution of eculizumab, based on the information in the specification and the skilled person's common knowledge at the filing date. Point (a) above is therefore fulfilled.

4.7 With regard to (b), evidence of the efficacy of eculizumab in treating PNH is provided in the examples of the patent specification. It is submitted that the evidence in the specification makes it more than plausible that the claimed composition would be useful in the treatment of PNH. The threshold test defined by T609/02 is therefore met.

4.8 It is further noted that neither Opponent has provided any evidence throughout the Opposition Division proceedings which demonstrates that any formulation of eculizumab as defined in claim 1 could not be produced by the skilled person. Nor has either Opponent provided any evidence that any formulation of eculizumab as defined in claim 1 would not be suitable in the treatment of PNH. The Opponents arguments in relation to sufficiency are therefore not substantiated in any way. Their arguments on sufficiency must accordingly fail.

5 Inventive Step

5.1 *D1 as Closest Prior Art and Differences from D1*

5.1.1 D1 was taken as closest prior art before the Opposition Division. The following features of claim 1 are not disclosed in D1:

(i) the sequence of eculizumab;
(ii) the composition of eculizumab, namely a single-use dosage form of a sterile, preservative-free solution comprising 300 mg eculizumab at a concentration of 10 mg/ml.

5.1.2 Difference (ii) relating to the composition features was accepted by both the Opposition Division and the Opponents. However, there was dispute before the Opposition Division regarding difference (i).

5.1.3 The Opposition Division in fact confirmed in their decision that D1 does not disclose the sequence of eculizumab as set out in SEQ ID NOs: 2 and 4 of the patent.

5.1.4 Nevertheless, the Opposition Division took the view that the term "eculizumab" as used in claim 1 was not restricted to the specific

antibody having a heavy chain as set out in SEQ ID NO: 2 and a light chain as defined in SEQ ID NO: 4. According to the Opposition Division, it also encompassed the antibody referred to as "eculizumab" in D1. In reaching this conclusion, the Opposition Division failed to properly interpret the claim of the patent in accordance with the description, as is required under Article 69 EPC.

5.1.5 The proper approach to patent claim interpretation, where questions arise as they do here, is to take account of the meaning given to a term in the description. In the present case, the patent defines the antibody referred to as "eculizumab" in paragraph [0103] by providing the "Eculizumab Heavy chain" sequence (SEQ ID NO: 2) and the "Eculizumab Light chain" sequence (SEQ ID NO: 4). The skilled person would therefore understand that "eculizumab" as recited in claim 1 is an antibody having the heavy chain sequence shown in SEQ ID NO: 2 and the light chain sequence shown in SEQ ID NO: 4.

5.1.6 Thus, the features of claim 1 which are not disclosed in D1 are:

(i) the sequence of eculizumab; and
(ii) the composition of eculizumab, namely a single-use dosage form of a sterile, preservative-free solution comprising 300 mg eculizumab at a concentration of 10 mg/ml.

5.2 *Objective Technical Problem*

5.2.1 The use of the claimed composition of eculizumab provides a low volume liquid formulation which is advantageous for IV administration, and which has a very good stability. The advantages of using a low volume liquid formulation for IV administration were explained in our submission of 22 March 2018 (see section 5.3.2 and onwards). In particular, providing a solution formulation avoids the need for reconstitution prior to IV administration. This reduces the risks associated with incomplete dissolution and avoids inconvenience for clinical staff. Further, the low volume of the composition means it is suitable for administration to a patient within an acceptable infusion time.

5.2.2 Regarding the improved stability of the composition, the Patentee has submitted data, in declaration D33, which demonstrates the improved stability, and reduced formation of aggregate, of the 10 mg/ml composition compared with a 30 mg/ml composition.

5.2.3 Figures 1 and 2 of D33 demonstrate that the percentage of eculizumab monomer in the composition decreases over time due to degradation of the antibody. Both the room temperature degradation (Figure 2) and the accelerated degradation (Figure 1, increased temperature) resulted in an approximately linear reduction of monomer content for both compositions studied. The rate of degradation of the 30mg/ml composition is greater than that of the 10 mg/ml composition, under both the storage conditions studied, and throughout the time period studied.

5.2.4 Dr Miano comments in his declaration that after 2 years storage at 2°C to 8°C, the 30 mg/ml composition would have a monomer content which has dropped below the threshold set for product release (below 98% monomer). A similar drop below the 98% monomer threshold occurs under accelerated conditions (at 23°C to 27°C) at six months. However, both Figure 1 and Figure 2 also show that reduction in the percentage monomer is observed not only after 2 years, but **from the outset** of the experiment. The rate of degradation of the 30 mg/ml eculizumab formulation is greater than that of the 10 mg/ml eculizumab formulation, at all time points. Thus, at any time point after make up of the composition, the percentage monomer remaining in the composition will be greater for a 10 mg/ml composition than for a 30 mg/ml composition.

5.2.5 The data of D33 therefore demonstrate that an eculizumab composition having a concentration of 10 mg/ml will have a greater monomer content, at the point at which it is administered to the patient, than a composition which is identical in all respects except for having a 30 mg/ml concentration. Since from a practical perspective there is always some delay between making up a composition and administering that same composition to a patient, the increase in percentage monomer in the 10 mg/ml eculizumab composition is of significant benefit to the patient, since it ensures a greater amount of the active substance is administered.

5.2.6 Opponent 2 raised a criticism of D33 during written proceedings before the Opposition Division, arguing that if the experiments were not side by side experiments, no reliable conclusions can be drawn because environmental factors may influence the results. D33 itself, however, dispels such criticism.

5.2.7 All of the experiments in D33 Figure 1 were carried out under controlled conditions which were identical in all cases. The same is true for the experiments reported in D33 Figure 2. Environmental factors do not have a significant effect, as is demonstrated by the consistency in results between identical experiments carried out on identical compositions.

5.2.8 If environmental factors had been significant, the skilled person would expect experiments carried out on the same composition to give differing results. However, this is not observed: In fact, both Figures 1 and 2 of D33 indicate results from multiple tests on the 10 mg/ml composition which show very little variation. On the other hand, where the composition is changed to a 30 mg/ml composition, a significant drop in stability is observed. It is clear that the drop in stability cannot be due to environmental factors. If it had been, similar levels of variation would have been observed between the multiple tests on the 10 mg/ml composition. The data presented in D33 therefore reliably shows an improvement in stability to aggregation for the 10 mg/ml composition compared to the 30 mg/ml composition.

5.2.9 Aggregation is well known to be disadvantageous in the context of a therapeutic antibody treatment. In particular, aggregation can lead to

immunogenicity and loss of efficacy. To demonstrate the effects of eculizumab aggregation, Dr Miano provided a second declaration, D54, which studied further the properties of the impurity produced on storage of an eculizumab composition. In the experiments described in D54, the impurity was isolated and studied by SEC (see Figure 1) and it was confirmed that a dimer of eculizumab had formed.

5.2.10 C5 binding activity studies showed that the activity of the dimer is approximately 50% of that of eculizumab itself. Thus, D54 demonstrates that a significant drop in potency occurs on aggregation of eculizumab to the dimer form.

5.2.11 The data of D33 and D54 collectively show that the delay between making up a composition of eculizumab, and administering that composition to a patient, can have a greater impact on the resulting treatment in the case of a 30 mg/ml composition, compared with a 10 mg/ml composition. The 10 mg/ml composition will retain a higher proportion of the intact monomer, and will accordingly retain a higher activity. Thus, the technical effect of the 10 mg/ml composition is to provide a highly stable composition of eculizumab, which minimises loss of activity in the period between when the composition is produced and when the composition is administered to the patient.

5.2.12 The objective technical problem which is solved by the invention is therefore the development of an antibody for use in the treatment of PNH, in a composition which is highly stable, and which can be provided in a low volume liquid formulation.

5.3 *Reformulation of the Objective Technical Problem*

5.3.1 The Opposition Division's decision indicated that the reformulation of the technical problem to that set out above was not allowable citing, *inter alia*, the fact that the stability of the antibody has no bearing on the patient and is therefore not related to the primary problem of the application as filed, namely treatments for PNH. The Patentee disagrees. Firstly, it is clear from the application as filed that storage of the antibody is necessary and thus maintaining the antibody in a suitable form for administration following a period of storage is required. Secondly, the minimisation of any aggregate in the composition which the patient actually receives is critical to ensure the safest and most effective treatment for the patient. In this way, the objective technical problem is not only implicit from the application as filed but is intimately connected with the problem of providing treatments for PNH.

5.3.2 It is the consistent jurisprudence of the Boards of Appeal that reformulation of the technical problem initially set out in the application as filed is allowable. Indeed, such reformulation is often necessary, for instance where it is discovered that the problem as set out in the application as filed is not solved across the scope of the claim.

5.3.3 Reformulation of the technical problem has been allowed (a) if the new technical problem can be deduced from the application as filed, (b) if the new technical effect can be deduced by the skilled person from the application in the light of the prior art, or (c) based on new effects, submitted during proceedings, where those new effects are implied by or related to the technical problem initially suggested.

5.3.4 The Guidelines (G-VII, 11) provide an example of (c), a new effect: where an application as filed relates to a pharmaceutical composition having a particular activity, new evidence relating to toxicity may be relied on in formulating the technical effect. This is the case even where toxicity is not mentioned at all in the application as filed, because toxicity and pharmaceutical efficacy would always be contemplated together. The general principle that new effects, submitted during prosecution, may be relied on to establish the objective technical problem is therefore well established.

5.3.5 In the case of the present patent, the problem to which the application as filed is addressed is the provision of a treatment for patients suffering from PNH, in particular using eculizumab. The application also provides compositions and kits suitable for such treatments (see final two paragraphs of page 3 of the PCT parent application).

5.3.6 It is apparent from the application as filed that the compositions for treating PNH which are described will be stored prior to delivery to the patient. In particular, the application as filed makes reference to a possible need for preservatives in the formulation (page 22, line 27). Preservatives would be unnecessary if the composition were not intended for storage. The kits described at page 26, line 21 onwards are clearly intended as sterile forms of storing the described composition. These kits may contain means for removing any preservative. It is therefore implicit in the description of the application as filed that the compositions described are intended to be stored before use, and moreover that they should be safe and effective (i.e. suitable for administration) after such storage.

5.3.7 Furthermore, the application as filed makes specific reference to a number of potential formulations for the described antibodies at page 24, lines 8 to 9 and 18 to 19. Of these, US 2003/0202972 is directed to "*stable formulations of modified antibodies*" (abstract) whilst US 2005/0158316 is directed to "*stable aqueous pharmaceutical formulation comprising a therapeutically effective amount of an antibody*" (see abstract). Furthermore, WO 03/106644 refers to "*Stabilized anti-respiratory syncytial virus antibody formulations*" (title) which exhibit "*stability, low to undetectable levels of aggregation*" (see abstract). WO 2004/066957 also describes formulations which "*exhibit stability, low to undetectable levels of aggregation*" (see abstract). Finally, WO 2004/091658 relates to antibody formulations which are "*stable*" (see abstract).

5.3.8 The composition which is claimed in the patent as granted is of course the specific composition disclosed in the application as filed, e.g. at page 22, lines 11 to 13, and differs from the compositions described in these cross-referenced documents. However, the fact that *all six* of the applications referenced in relation to possible formulations are specifically concerned with providing highly stable antibody formulations is a clear indication to the skilled reader that when considering eculizumab compositions, stability is an important problem to be considered.

5.3.9 Thus, the need for highly stable formulations is clear from the application as filed from (a) the need for storage of compositions and kits prior to their use in therapy, and (b) the examples of suitable formulations which were developed in order to provide improved stability.

5.3.10 Furthermore, it would be clearly recognised by one of skill in the art as desirable to provide a formulation of eculizumab where aggregation is kept to a minimum so that the PNH treatment is as efficacious as possible. The improvement in stability directly affects the amount of eculizumab monomer which is actually administered to the patient, and thus may affect the ability of the composition to optimally treat PNH. This is particularly relevant in the present case where the dimer form of eculizumab has only 50% of the activity of the monomer form (as evidenced by D54). A change from monomer to dimer on storage thus reduces the efficacy of the composition in treating PNH. The objective technical problem of improved stability is thus clearly related to the problem of providing optimally effective treatments for PNH.

5.3.11 The Opposition Division, on the contrary, found that the problem of improved stability is not related to the claimed therapeutic use and therefore the problem could not be reformulated by taking into account the stability data of D33. Reasoning for this aspect of the decision is set out at page 12 of the decision. The Opposition Division considers that long term stability is an important factor in producing a commercial composition. However, the Opposition Division does not consider that stability is related to the treatment of PNH. In the third paragraph of page 12 of the decision, the Opposition Division argues that the stability of the composition has no bearing on the patient. This is not correct: the presence or absence of aggregates in the composition which is administered can have a significant bearing on the patient.

5.3.12 Firstly, as explained above, it is not the case that the 10 mg/ml and 30 mg/ml compositions tested by Dr Miano were equivalent for a period of time, with the 30 mg/ml composition degrading only after prolonged storage. Rather, the differences in stability are apparent throughout the test period. Thus, even for a short period of storage of the drug before administration (which the Patentee submits is inevitable as the drug will not be manufactured at the bedside of the patient), the 10 mg/ml composition shows improved stability to aggregation. This means that the problem of aggregation is not one which only affects commercial production considerations: on the contrary, the problem of aggregation is

relevant to the patient in all cases.

5.3.13 Secondly, the aggregated eculizumab has been shown, in D54, to have only 50% of the binding activity of eculizumab in monomer form. Thus, this improved stability to aggregation which is demonstrated by the 10 mg/ml composition can have a significant bearing on the patient as it means the drug they receive will have a higher proportion of the active monomeric form.

5.3.14 The Opposition Division was therefore wrong to discount the evidence of D33 and D54. These data should be taken into account in formulating the objective technical problem.

5.4 *Technical Problem is Solved Across the Scope of the Claim*

5.4.1 The Opponents asserted during the oral proceedings before the Opposition Division that the claimed composition is not inventive because the technical problem of improved stability with respect to aggregation is not solved across the scope of the claim. The Opponents have not provided any evidence to support this assertion. The Opponents' assertion is made on the basis of the general uncertainties in the formulation of antibody drugs.

5.4.2 To address this assertion, further experimental data is here submitted as D55. D55 is a third declaration of Dr Dino Miano, in which stability studies are described of nine different eculizumab compositions. These compositions include three different sets of formulation excipients, and three different pH levels. Composition F2 corresponds to Soliris®, the formulation tested in D33 and D54. F1 and F3 are identical compositions except for variation in the pH. The formulation excipients in compositions F4 to F9 differ from those used in F1 to F3. For instance, F4 to F6 do not contain any surfactant. Further, F4 to F9 use a histidine buffer rather than a phosphate buffer, and each of F2, F5 and F8 vary in terms of their osmolality (ionic strength).

5.4.3 For each of the nine different compositions, Dr Miano studied the formation of aggregates in both a 10 mg/ml composition and a 30 mg/ml composition. These experiments were carried out in the same temperature controlled unit and thus each composition was subject to identical conditions. In each case, Dr Miano found the 10 mg/ml composition to be more stable to aggregation than the corresponding 30 mg/ml composition having the same formulation and pH.

5.4.4 D55 therefore demonstrates that the objective technical problem (providing an antibody, in a composition which is highly stable, and which can be provided in a low volume liquid formulation) is solved by use of a 10 mg/ml composition for a wide range of different compositions, including those having varying pH and formulation excipients. The Opponents have provided no evidence whatsoever to support their

argument that the claim is overly broad. Under these circumstances, it is considered that D55 is sufficient evidence to demonstrate that the technical problem is solved across the full scope of the claim.

5.5 *Use of Claimed Sequence is not Obvious*

5.5.1 The solution to the objective technical problem is the provision of eculizumab (i.e. an antibody having the heavy and light chain sequences set out in SEQ ID NOs: 2 and 4, respectively), in a composition as defined in claim 1. This solution would not have been obvious to the skilled person starting from D1.

5.5.2 D1 identifies eculizumab only by reference to D17 (US Patent No. 6,355,245) and D13 (Thomas et al. (1996)). The relevant disclosure in D1 is as follows:

> "*Particularly useful anti-C5 antibodies are h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1. Methods for the preparation of h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1 are described in U.S. Pat. No. 6,335,245 and "Inhibition of Complement Activity by Humanized Anti-C5 Antibody and Single Chain Fv", Thomas et al., Molecular Immunology, Vol. 33, No. 17/18, pages 1389-1401, 1996, the disclosures of which are incorporated herein in their entirety by this reference. The antibody h5G1.1-mAb is currently undergoing clinical trials under the tradename eculizumab.*"(D1, page 5, left column last 4 lines to right column, line 8).

5.5.3 Neither D17 nor D13 mentions "eculizumab." D17 and D13 describe the cloning of murine antibody 5G1.1 and the construction of humanised variable regions and humanised antibody fragments.

5.5.4 As noted by the Opposition Division, D17 does not give a complete sequence of a humanised 5G1.1 antibody.

5.5.5 D13 also fails to provide complete heavy and light chain sequences of a full length antibody. In Figure 1 of D13, VL and VH sequences of two humanised versions of the 5G1.1 antibody are shown. D13 discloses CDR grafted VL and VH sequences (h5G1.1VHC and h5G1.1VLC in Figure 1 of D13) and also VL and VH sequences comprising back mutations (h5G1.1VHC+F and h5G1.1VLC+F in Figure 1 of D13). D13 reports that an intact humanised antibody of the IgG4 isotype was constructed and produced (D13, page 1396, paragraph spanning columns).

5.5.6 D13 explains why the IgG4 isotype was chosen for the full length antibody, reporting that:

> "*The human IgG4 isotype was chosen, as this isotype does not activate human complement (Tao et al., 1993) and there is only one known allotype of IgG4 (Ghanem et al., 1988), precluding the potential*

development of allo-antibodies in patients. The humanized h5G1.1 (CDR) HuG4 antibody bound to human C5 with a similar avidity as the murine antibody when assayed by ELISA (Fig. 9) and inhibited lysis of porcine aortic endothelial cells as effectively as the murine antibody, with a 1:1 molar ratio of antibody binding sites to human C5 being sufficient for inhibition (Fig. 10)" (D13, page 1399, right column, second paragraph, lines 15-25).

5.5.7 As D1 does not include any sequence information for eculizumab, based on this disclosure of D13, at the filing date of the patent, the skilled person would understand that the "*antibody h5G1.1-mAb*" that "*is currently undergoing clinical trials under the trade name eculizumab*" as referred to in D1 at page 5, right column, lines 6-8 has human IgG4 constant region sequences. Eculizumab, however, is not an IgG4 isotype antibody, but has a unique, non-naturally occurring, protein engineered heavy chain having the sequence shown in SEQ ID NO: 2 of the patent and the light chain sequence shown in SEQ ID NO: 4 of the patent (see paragraphs 4 and 5 of D57, a declaration of Leonard Bell filed at the USPTO in connection with a corresponding US patent application).

5.5.8 As eculizumab did not receive marketing approval until after the filing date of the patent (see paragraph 14 of D58, a declaration of Laural Boone filed at the USPTO in connection with a corresponding US patent application), it was not commercialised at the filing date. Eculizumab used in the clinical trials reported before the filing date was also not available to the public. The trials were carried out under confidentiality agreements with Alexion (see paragraphs 7-9 and 12-13 of D58; and paragraph 2 of D57), the storage, use and disposal of unused eculizumab was strictly controlled (see paragraphs 10-11 of D58; and paragraph 2 of D57), and the clinical study protocols did not inform clinicians and patients involved in the trial of the sequence of eculizumab (see paragraph 6 of D58). In addition, the patients did not have access to eculizumab which was administered in the clinic by intravenous infusion (see, for example, D1, page 9, paragraph [0082] and paragraph 2 of D57).

5.5.9 Therefore, the skilled person wishing to use eculizumab would have needed to produce it himself based on the teaching in D1/D13. In following this teaching he would have made an IgG4 isotype antibody, and not an antibody having the heavy and light chain sequences shown in SEQ ID NOs: 2 and 4.

5.5.10 It would not have been obvious to a skilled person following the teaching of D1 to provide a composition of an antibody having the engineered heavy chain sequence shown in SEQ ID NO: 2 of the patent and the light chain sequence shown in SEQ ID NOs: 4 of the patent for treating PNH.

5.5.11 D1 describes a study wherein PNH patients were treated with the antibody, "eculizumab". D1 describes the antibody by pointing to D13 which discloses an IgG4 isotype antibody and does not disclose SEQ ID NOs: 2 and 4 of the patent. The initial four week study was successful and so was extended to a total of 2 years for 10 out of the 11 original

patients. The effects of the treatment on different physiological parameters and symptoms involved in PNH were analysed in D1, which reports that the treatment was successful.

5.5.12 As discussed above, the skilled person reading D1 would, in the absence of any sequence information in D1, understand that "eculizumab" was the humanised IgG4 isotype antibody disclosed in D13 that is explicitly referenced in D1. In contrast, SEQ ID NO: 2 does not include a human IgG4 constant region sequence, but rather has a unique, non-naturally occurring, engineered constant region sequence.

5.5.13 Thus, when looking to solve the problem of providing an antibody for use in the treatment of PNH, in a composition which is highly stable, and which can be provided in a low volume liquid formulation, the skilled person would have provided a composition of the IgG4 isotype antibody of D13 that D1 identified as "eculizumab". The skilled person would have had no motivation to use an antibody different than the one described in D1 in view of the report in D1 of the successful treatment of PNH, and the teaching in D13 discussed above that the human IgG4 isotype has the advantages that it does not activate human complement and has only one known allotype, precluding an immune response against the constant region of the antibody.

5.5.14 In particular, the skilled person would not consider using a different, engineered constant region sequence in place of the IgG4 isotype antibody disclosed in D13 because he would be concerned about the immunogenicity of the therapeutic antibody and D13 teaches that the IgG4 isotype is ideal in this regard. Also, at the filing date of the patent, other IgG4 isotype antibodies had been commercialised as therapeutic antibody products (see D20, table 1). Significantly, at the filing date of the patent, no antibody having the uniquely-engineered heavy chain constant region of SEQ ID NO: 2 was on the market or even known to be studied in human clinical trials.

5.5.15 Accordingly, based on the teaching in D1 and D13, the skilled person would not have tried to provide a composition of an antibody having the heavy chain sequence set out in SEQ ID NO: 2 of the patent and the light chain sequence set out in SEQ ID NO: 4 of the patent for treating PNH.

5.5.16 No other prior art would have motivated the skilled person to diverge from the teaching of D1. All of the cited prior art documents that describe clinical trials using eculizumab either contain no sequence information relating to "eculizumab," or define "eculizumab" with reference to only one or both of the documents cited as D13 and D17 in the present proceedings.

5.6 *Use of Claimed Composition is not Obvious*

5.6.1 Regarding the use of a 10 mg/ml composition, there is nothing in the prior art which would suggest to the skilled person that the 10 mg/ml

composition would be beneficial, let alone that this composition would solve the objective technical problem of developing an antibody in a composition which is highly stable, and which can be provided in a low volume liquid formulation. The Opposition Division considered that the teaching of D22 would lead the skilled person to the 10 mg/ml composition. However, the Opposition Division erred in their reasoning in a number of respects.

5.6.2 D22 is concerned with antibodies which can be used to prevent or treat a pulmonary disease or condition (see abstract and paragraph [0011]). D22 is not concerned with treating PNH.

5.6.3 Further, D22 is specifically concerned with formulating antibodies for administration directly to the lung, by inhalation. This may be by inhalation of an aerosolised composition (paragraph [0006]), or by nebulisation (paragraph [0007]). D22 discloses that examples of useful antibodies "*include anti-C5 antibody or antibodies that inhibit activation of the complement cascade, for example the antibodies described in U.S. Pat. No. 6,355,264*", and that "*Certain preferred embodiments employ pexelizumab or eculizumab as the antibody therapeutic*" (paragraph [0004]). The sequence of eculizumab is not disclosed in D22. The only mention of a formulation containing "eculizumab"[1] is under the heading "Nebulisation Formulations" at paragraph [0169]. There is no mention of systemic administration of eculizumab in D22. In fact, paragraph [0004] (see the passage spanning the two columns of page 1) indicates that the methods may specifically be for administration *other* than systemically. In contrast, treatment of PNH requires compositions for IV administration.

5.6.4 Paragraph [0169] onwards refers to a specific formulation which is in accordance with Formulation 1 and has an "eculizumab" content of 30 mg/ml (paragraph [0171]). Notably, however, Formulation 1 is not described as a solution.

5.6.5 As is well known in the art, and discussed for instance at paragraph [0074] of D22, a formulation for nebulisation may be a solution, suspension or slurry. Indeed, budesonide is commonly administered by nebulising a suspension (as noted in paragraph [0062] of D22, with reference to the drug Pulmicort). Therefore, the lack of any mention that the formulation is a solution is significant in the context of a formulation for nebulization. Paragraph [0171] of D22 does not recite a 30 mg/ml "*solution*", and the Opposition Division was wrong to assume the 30 mg/ml formulation to be a solution simply because it is intended for nebulization; it could be a slurry or suspension.

5.6.6 There is further reference at paragraph [0173] to nebulisation of a formulation of "eculizumab." SDS-PAGE and HPLC analyses are carried out following nebulisation. However, this paragraph does not indicate

[1] D22 does not disclose what "eculizumab" is, nor does it provide any sequence for "eculizumab". This point is discussed further at section 5.6.17

the nature of that formulation, merely referring to it as "a suitable formulation."

5.6.7 Despite these differences, the Opposition Division considered that D22 was relevant to the present invention since it is the only document actually describing concentrations of eculizumab in liquid compositions. In determining whether D22 is relevant to the present invention, the Opposition Division considers that the question to be answered is "what must be the concentration of eculizumab in a solution for said solution to be suitable to be administered to a patient" (see page 10 of the Decision). The Patentee considers that this is not the correct question.

5.6.8 The present invention is concerned with providing compositions of eculizumab which are *suitable for treating PNH* (which, as is known from D1, is administered systemically via IV). The skilled person would therefore be trying to answer the question of what composition would be suitable for treating PNH, and would thus be interested in formulations for IV administration. More particularly, the skilled person would be concerned with compositions which would solve the objective technical problem, i.e. compositions for use in the treatment of PNH which can be provided as a low volume liquid composition and have good antibody stability. D22 would be taken into account by the skilled person only to the extent that it assists in answering this question.

5.6.9 The Opposition Division concluded that D22 would be relevant to the present invention, citing in its reasoning that: "As a matter of fact, [D22] is an indication that solutions of eculizumab at these concentrations have adequate properties to be administered to a patient in terms of safety and efficiency". However, D22 provides no indication that the formulations of eculizumab referred to at paragraphs [0171]-[0172] are solutions. Moreover, as discussed below, D22 provides no indication that the formulations of eculizumab described would be safe and efficacious *for treating PNH,* or indeed for administration by IV.

5.6.10 Firstly, as acknowledged by the Opposition Division, the indication which is to be treated in D22 is very different from that of the present invention. D22 aims to deliver drug to the lungs to treat pulmonary disease and specifically notes that administration may be non-systemic. In contrast, the present invention aims to treat PNH via a systemic administration. It cannot be assumed that the same composition will be safe and efficacious for both.

5.6.11 Secondly, there are fundamental differences in the means of administration described. For instance, during nebulisation, only a proportion of the nebulised material is actually inhaled by the patient. The amount of drug which leaves the nebuliser and is available for inhalation by the patient is indicated by the "nebuliser efficiency". In D22, the nebuliser efficiency ranges from 11.24 to 52.46 % (see Figure 13). Further, of those particles which exit the nebuliser, only a proportion are within the "respirable fraction", i.e. the fraction of drug which reaches the alveoli and accordingly has a therapeutic effect on the

patient. D22, Figure 13 also indicates the respirable fraction, which ranges from 44.91 to 74.21%. Thus, only a relatively low amount of the drug which is nebulised actually therapeutically affects the patient. In contrast, administering the same drug via IV will cause 100% of the drug to enter the patient's blood stream.

5.6.12 The Opposition Division's conclusion regarding the relevance of D22 is therefore based on a false premise, since D22 would not lead the skilled person to understand that 1-30 mg/ml solutions of eculizumab would be safe and efficacious *for treatment of PNH by IV administration*.

5.6.13 In fact, the considerations which the skilled person would be concerned with when seeking a suitable composition for IV administration are very different from those which would be considered for a nebulised formulation. For nebulisation, important factors are the size of the particles produced, since this influences the respirable fraction of the drug, and the ability of the drug to withstand the nebulisation process.

5.6.14 On the other hand, for IV administration, a much more important consideration is whether the drug can be provided in solution form, without particulate matter present. For this reason, there is much incentive to provide a liquid formulation which avoids the risks of incomplete dissolution by a clinician, and which is stable in solution form. Such a requirement is not present for a nebulised formulation, which can be administered as a suspension or slurry. Similarly, the burden on the patient of high volume doses delivered via IV can be problematic and leads to poor patient compliance in connection with repeat treatments. Low volume is therefore also an important factor for an IV administered drug. Again, this factor is not important for nebulisation.

5.6.15 These different considerations lead, in practice, to drugs being formulated in very different ways for the two administration routes. Salbutamol, for instance, is commonly administered via nebuliser at a concentration of 2 mg/ml. In contrast, when this drug is administered via IV, a very different concentration of 0.5 mg/ml or 1 mg/ml is used (see D60, page 3).

5.6.16 The skilled person aware of the D22 disclosure, therefore, would not consider that this helps them to determine whether the eculizumab formulations described are safe and efficacious *for treating PNH*. Compositions for treating different diseases, via different routes of administration, require entirely separate study. For this reason, D22 is not relevant to the question which the skilled person would need to answer in connection with the present invention, i.e. what compositions may be suitable for treating PNH. D22 would not therefore be taken into account by the skilled person.

5.6.17 Moreover, D22 makes no reference to an antibody having the heavy chain shown in SEQ ID NO: 2 and the light chain shown in SEQ ID NO: 4, nor to the ability of the skilled person to provide a composition of that antibody in a highly stable solution at low volume. Regarding the antibody

disclosed in D22, D22 refers to "eculizumab" in general terms but does not provide any sequence. It refers to the US patent referred to in the present proceedings as D17 as providing examples of anti-C5 antibodies, but as discussed above D17 does not refer to "eculizumab" and does not disclose SEQ ID NOs: 2 and 4 of the patent (see paragraph [0004]).

5.6.18 Regarding the "eculizumab" compositions, there is no mention of any means to improve the stability of the compositions, and no mention that this can achieved at a concentration of 10 mg/ml. Indeed, the only specific composition exemplified in D22 is that at paragraph [0171] which has a concentration of 30 mg/ml. In this respect, D22 teaches away from the improved compositions now claimed.

5.6.19 Accordingly, D22 does not assist the skilled person in finding a solution to the objective technical problem. Even if the skilled person had considered D22, it would not have led them to produce the claimed composition, in expectation of achieving a high stability, low volume solution of eculizumab.

5.6.20 The claimed subject matter is therefore inventive over D1 combined with D22.

5.7 *Other Documents Relied on by the Opponents: D20, D21*

5.7.1 The Opponents argued, during the Opposition Procedure, that the 10mg/ml composition of eculizumab would be obvious to the skilled person based on the common general knowledge, as represented by D20 and D21. Detailed comments on this argument were provided in our submissions of 22 March 2018 (see Section 5.5) and 21 November 2018 (see Section 3). The issues are summarised in brief here.

5.7.2 D20 is a mini review of antibody formulation and makes clear, in the abstract, the difficulties in providing viable antibody pharmaceuticals due to their unpredictable solution behaviour. Table 1 at pages 2 to 4 of D20 demonstrates the wide variety of differing conditions which have been used in the past to produce antibody products. Notably, this table only shows those antibody products which have been commercially approved. A much greater number of antibodies have been developed but have not have been approved, in many cases due to difficulties in producing a suitable formulation (as explained by Dr Klibanov in his declaration D39, paragraph 5).

5.7.3 Many antibodies cannot be formulated as a liquid at all, since they lack the required stability. This is explained by Dr Klibanov in D39, paragraph 6 and is also confirmed in D20 at the top left-hand column of page 18 ("*like most proteins, some antibodies are not stabilised enough in a liquid form and lyophilized forms will have to be considered*").

5.7.4 Turning to D21, this document is a further review of the formulation of antibody therapeutics. In the abstract, the difficulties and technical challenges in the formulation of antibodies is highlighted. In the right-hand column of page 688 (see the end of the penultimate paragraph) D21 mentions that the majority of work on antibody formulation has been carried out on IgG1 antibodies. Page 689 goes on to discuss the preliminary work which has been carried out on other types of antibodies, including engineered antibodies. It is noted that there is little information on formulation approaches for such antibodies (of which eculizumab is an example).

5.7.5 As discussed in the submission of 22 March 2018 (section 4, in particular 4.2.4), eculizumab has a unique engineered structure. No analogously-engineered antibodies were even in development at the time of filing the present application. There was certainly no guidance available in the common general knowledge as to how such antibodies should be formulated. This lack of knowledge in formulating engineered antibodies is particularly highlighted by D21 at page 689.

5.7.6 Thus, the common general knowledge at the time of the invention, as evidenced by D20 and D21, did not provide the skilled person with a straightforward route to providing a composition of eculizumab for treating PNH. There was, of course, no information at all in the common general knowledge about the formulation of eculizumab, as its unique engineered structure was not at the time known.

5.7.7 The Opponents suggest that, starting from D1, it would be obvious to the skilled person to produce a composition of eculizumab in solution form at 10mg/ml, because this is a "typical" antibody formulation. The Opponents further suggest that the skilled person would simply try the 10mg/ml solution and see if it works. However, these suggestions are made with the benefit of hindsight.

5.7.8 Firstly, neither D1 nor D20/D21 discloses the sequence of eculizumab. Nor would the claimed sequence have been obvious from these disclosures, for the reasons set out above.

5.7.9 Secondly, at the time of the invention, even if the skilled person had developed eculizumab having the sequence set out in the patent (which the skilled person would not have done for the reasons described above), he/she would not have been aware as to whether a solution formulation of eculizumab would be feasible at all, let alone whether 10mg/ml would provide a high stability, low volume composition suitable for IV administration. Indeed, D20 and D21 are evidence of the wide array of different types of compositions (including lyophilized compositions) which had been used in the past for different antibodies. They provide no guidance in relation to eculizumab itself.

5.7.10 There is therefore nothing in the common general knowledge which would lead the skilled person to the claimed composition, with reasonable expectation that it would provide the desired highly stable, low volume

solution composition for use in treating PNH. The claimed subject matter is inventive over D1 combined with the common general knowledge.

5.8 *D29*

5.8.1 The Opponents have also argued that the claimed subject matter would be obvious over D1 combined with D29. This matter was addressed in detail in our submission of 22 March 2018, Section 5.9.

5.8.2 In brief, D29 would not be of assistance to the skilled person in seeking a suitable composition for eculizumab. D29 is primarily related to formulations of anti-CD18 and anti-CD20 antibodies. There is no reference to eculizumab at all. The skilled person would have had no information to assist him/her in developing the antibody which is now claimed. Further, even if this particular antibody had been developed (which would not have been done for the reasons discussed above), the skilled person would have had no expectation that the formulations described in D29 would be suitable for eculizumab. This is particularly the case given the knowledge in the art at the time (as evidence by D20 and D21) that even similarly structured antibodies may have very different requirements as to how they can be suitably formulated. Eculizumab is an engineered antibody and is NOT structurally similar to the antibodies described in D29.

5.8.3 The Opponents have particularly referred to column 22, lines 15 to 17 of D22 as an exemplary concentration for an antibody composition. However, although a variety of different concentrations are mentioned here, it is clear that the actual concentration used for any particular antibody would need to be selected on a case by case basis. For instance, the examples of D29 relate to an anti-CD18 antibody which is formulated at a concentration of 1mg/ml (see example 1, column 29 table 5) and an anti-CD20 antibody which is formulated at 40mg/ml (see example 2, column 40).

5.8.4 Moreover, there is nothing in D29 which points to any solution to the objective technical problem and no reference to providing a stable composition of eculizumab. Accordingly, starting from D1 and seeking a composition of eculizumab which combines the benefits of good stability and low volume liquid formulation, D29 would not be of assistance to the skilled person. It certainly would not direct them to the claimed 10mg/ml composition in the expectation that it would provide the desired highly stable, low volume solution composition.

5.8.5 The claimed subject matter is therefore inventive over D1 combined with D29.

6 Auxiliary Requests

6.1 Should the Board of Appeal decide that the main request is not allowable, the Patentee requests maintenance of the patent on the basis of one of Auxiliary Requests 1 to 8, as discussed below.

6.2 *Auxiliary Request 1*

6.2.1 In Auxiliary Request 1, the sequence of eculizumab is inserted into the claim in order to clarify that reference to "eculizumab" in claim 1 is that defined by SEQ ID NOs: 2 and 4. Basis for the amendment can be found in paragraph [0020] of the application as filed, as well as SEQ ID NOs: 2 and 4, labelled eculizumab heavy chain and eculizumab light chain, respectively.

6.3 *Auxiliary Request 2*

6.3.1 Auxiliary Request 2 is identical to Auxiliary Request 1, except that the light chain of eculizumab is defined as consisting of residues 23 to 214 of SEQ ID NO: 4. Thus, the amino acids corresponding to the leader sequence (residues 1 to 22 of SEQ ID NO: 4) are excluded.

6.3.2 It would have been immediately apparent to the skilled person that the eculizumab light chain sequences shown in SEQ ID NOs: 3 and 4 of the application for the patent include a 22 amino acid signal peptide at the N-terminus that would be cleaved off during antibody production in the cell, and consequently that the recitation at page 5, lines 30-33 of the application of an antibody that comprises a light chain that "consists of SEQ ID NO:4" is an error. The skilled person would immediately have known that it was the applicant's intention to define the antibody as including only the mature light chain sequence, i.e. residues 23 to 214 of SEQ ID NO:4.

6.3.3 This is evidenced by D59, a declaration of Dr Bruce Andrien, and was acknowledged by the Opposition Division at paragraph 2.3.1 of the Decision.

6.4 *Auxiliary Request 3*

6.4.1 Auxiliary Request 3 corresponds to the main request, but specifies that the pH of the composition is 7.0. Basis for this amendment can be found at paragraph [0090] of the application as filed. Auxiliary Request 3 relates to a narrower scope of composition and is filed to address the Opponents' assertion that the claim scope of the patent as granted is overly broad, and that high stability has not been achieved across the scope of the claim.

6.5 *Auxiliary Request 4*

6.5.1 Auxiliary Request 4 combines the amendments of Auxiliary Requests 1 and 3.

6.6 *Auxiliary Request 5*

6.6.1 Auxiliary Request 5 combines the amendments of Auxiliary Requests 2 and 3.

6.7 *Auxiliary Request 6*

6.7.1 Auxiliary Request 6 corresponds to Auxiliary Request 1 before the Opposition Division.

6.8 *Auxiliary Request 7*

6.8.1 Auxiliary Request 7 combines the amendments of Auxiliary Requests 6 and 3.

6.9 *Auxiliary Request 8*

6.9.1 Auxiliary Request 8 corresponds to Auxiliary Request 2 before the Opposition Division.

7 **Summary**

7.1 In conclusion, the Opposition Division's decision should be set aside and the patent maintained as granted.

IN THE MATTER OF
European Patent No. EP 2 359 834

DECLARATION OF DR. DINO MIANO

I, Dr. Dino Miano, hereby declare as follows:

1. I am Dr. Dino Miano of Alexion Pharmaceuticals, Inc., 100 College St, New Haven, Connecticut, USA. I hold a Ph.D. degree in Chemistry and Biochemistry from the University of Massachusetts, and a Bachelor of Science degree in Chemistry and Biology from the University of Hartford. I have been employed by Alexion Pharmaceuticals for over 20 years, and I currently hold the post of Executive Director, Global Analytical & Pharmaceutical Development. Prior to this I was employed by R.W. Johnson Pharmaceutical Research Institute in Raritan, New Jersey for approximately two years. During my employment at Alexion my responsibilities and work have focused on analytical sciences and protein characterization. I have significant expertise in formulation development and analysis, which includes designing, performing and overseeing experiments that assess the impact of protein concentration, formulation, and storage conditions on the integrity and stability of a protein over time.

2. I have submitted two declarations in the proceedings before the Opposition Division: (1) a March 17, 2018 declaration showing that the 10 mg/mL commercial formulation of eculizumab has superior stability to an otherwise-identical 30 mg/mL eculizumab formulation, as measured by percent monomer (i.e., aggregation) when tested over six months when stored at both 2-8C and 23-27C (D33 in these proceedings); and (2) a January 14, 2019 declaration showing that there is a significant decrease in the potency of eculizumab upon its aggregation into the dimer form (D54 in these proceedings). A copy of my resume was provided as Exhibit 1 with my first declaration, D33.

3. As explained in my two previous declarations, the percent aggregation of eculizumab, as determined by gel permeation high performance liquid chromatography, a stability indicating assay, is one of the parameters used to ensure that the formulation of eculizumab which is provided to clinicians, and used to treat patients, has a high content of eculizumab monomer and a low content of aggregate. As demonstrated in my January 14, 2019 declaration, D54, the percent of aggregation is a critical measure of stability given that greater aggregation leads to less percent monomer, more dimer and thus significantly lower drug potency, as well as potentially increased immunogenicity.

4. We conducted stability experiments to determine the effect of eculizumab concentration (10 mg/mL versus 30 mg/mL) on the stability of eculizumab for compositions having varying formulation excipients and pH. The nine formulations that were tested (designated F1 through F9), each at both eculizumab concentrations of 10 mg/mL and 30 mg/mL, are shown in Table 1 below.

Table 1. Description of three different eculizumab formulations tested at three different pH values.

F1	10 mM phosphate, 150 mM NaCl, 0.02% PS-80 pH 6.5
F2	10 mM phosphate, 150 mM NaCl, 0.02% PS-80 pH 7.0
F3	10 mM phosphate, 150 mM NaCl, 0.02% PS-80 pH 7.5
F4	20 mM histidine, 50 mM serine, 3% sorbitol, 1.5% mannitol pH 6.5
F5	20 mM histidine, 50 mM serine, 3% sorbitol, 1.5% mannitol pH 7.0
F6	20 mM histidine, 50 mM serine, 3% sorbitol, 1.5% mannitol pH 7.5
F7	10 mM histidine, 10% trehalose, 0.01% PS-20 pH 6.5
F8	10 mM histidine, 10% trehalose, 0.01% PS-20 pH 7.0
F9	10 mM histidine, 10% trehalose, 0.01% PS-20 pH 7.5

5. The osmolalities of the formulations were determined as:
Formulation F2: 303 mOsm/kg;
Formulation F5: 336 mOsm/kg; and
Formulation F8: 319 mOsm/kg.

I would not expect the pH of the composition to affect the osmolality of the composition. It can generally be assumed that the osmolality is dependent on the formulation excipients but not on the pH.

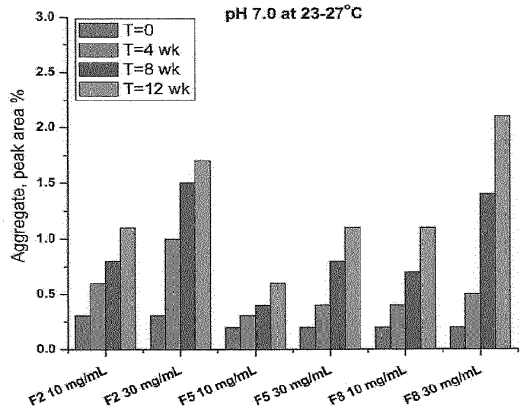
6. The experiments were carried out over a 12 week period, using accelerated conditions (storage at 25°C over 12 weeks). Use of accelerated storage conditions is a recognized way to reveal inherent differences in stability between different compositions.

7. The compositions were analysed using both SEC-HPLC (which shows the amount of aggregate formed) and icIEF (which shows the percentage of acidic isoforms present). The detailed protocol that we followed is attached as Exhibit 1. We did not detect any difference between the acidic isoforms generated by the 10 mg/mL and 30 mg/mL compositions in this study. However, there were differences in the amount of aggregate formed, as demonstrated by the SEC-HPLC results.

8. The results show that regardless of the formulation excipients or the pH, the 10 mg/mL eculizumab concentration consistently has significantly lower aggregation (i.e., improved stability) as compared to the corresponding 30 mg/mL eculizumab formulation. Specifically, Figure 1 shows that for each of the formulations studied at pH 7.0 (F2, F5 and F8 listed above), the 10 mg/mL eculizumab concentration formulations had significantly lower aggregation than the corresponding 30 mg/mL eculizumab concentration formulations when stored at 25°C for 12 weeks.

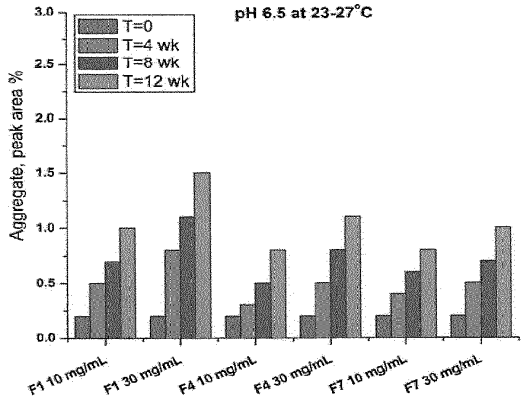
Samsung Bioepis Exhibit No. 1002
Page 14009

Figure 1. Formulations F2, F5 and F8 (all at pH 7.0) show lower aggregation at 10 mg/mL eculizumab as compared to their 30 mg/mL eculizumab counterparts.



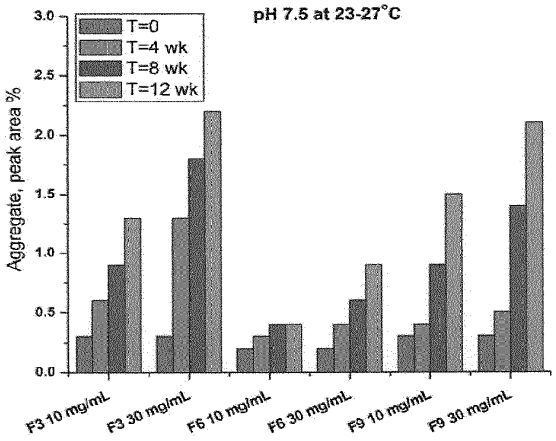
9. Figures 2 and 3 below show the same trend for the formulations at pH 6.5 and pH 7.5: each formulation at pH 6.5 shows better stability at 10 mg/mL eculizumab than 30 mg/mL eculizumab, and each formulation at pH 7.5 also shows better stability at 10 mg/mL eculizumab than 30 mg/mL eculizumab.

Figure 2. Formulations F1, F4 and F7 (all at pH 6.5) show lower aggregation at 10 mg/mL eculizumab as compared to their 30 mg/mL eculizumab counterparts.



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Figure 3. Formulations F3, F6 and F9 (all at pH 7.5) show lower aggregation at 10 mg/mL eculizumab as compared to their 30 mg/mL eculizumab counterparts.



10. These results demonstrate that regardless of the different excipients used in the formulation or the pH of the formulation, the 10 mg/mL eculizumab concentration consistently exhibits less aggregation and thus improved stability as compared to the 30 mg/mL concentration. This is important because, as discussed above, greater aggregation is associated with decreased potency of the drug and potentially higher immunogenicity.

I believe and do hereby declare that the facts stated above are to the best of my knowledge true.

Dr. Dino Miano

21 June 2019
Date

Samsung Bioepis Exhibit No. 1002
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Study Plan

Study Information

Title: Evaluation of the Effect of Eculizumab Concentration on Monoclonal Antibody Stability in the pH range 6.5 to 7.5 in Three Different Formulations

Scope and Study Objectives

The scope of this project encompassed the evaluation of the effect of eculizumab concentration (10 mg/mL and 30 mg/mL) on the monoclonal antibody stability in the pH range 6.5 -7.5 in three different formulations on 12 weeks accelerated (23-27°C) stability, as analyzed by two stability-indicating assays, SEC-HPLC and icIEF.

Introduction

Soliris®(eculizumab) is manufactured as liquid product in PBS formulation, comprising 10 mM sodium phosphate, 150 mM NaCl, 0.02 % PS-80 at pH 7.0 at a 10 mg/mL monoclonal antibody concentration. The current study was aimed at evaluating the effect of the concentration of eculizumab (10 mg/mL versus 30 mg/mL) on antibody stability, as assessed by two stability-indicating assays, SEC-HPLC and icIEF, in three different formulations at three different pH levels. The study was performed for 12 weeks using eculizumab in these nine different formulations at two different concentrations (see Table 1 below). The samples were incubated alongside one another at 23-27°C for 0 weeks, 4 weeks, 8 weeks and 12 weeks.

Table 1. F1 through F9 below are the nine formulations tested in this study using eculizumab concentrations of 10 mg/mL and 30 mg/mL for each formulation.

Formulation No.	Formulation excipients and pH
F1	10 mM phosphate, 150 mM NaCl, 0.02 % PS-80 pH 6.5
F2	10 mM phosphate, 150 mM NaCl, 0.02 % PS-80 pH 7.0
F3	10 mM phosphate, 150 mM NaCl, 0.02 % PS-80 pH 7.5
F4	20 mM histidine, 50 mM serine, 3 % sorbitol, 1.5 % mannitol pH 6.5
F5	20 mM histidine, 50 mM serine, 3 % sorbitol, 1.5 % mannitol pH 7.0
F6	20 mM histidine, 50 mM serine, 3 % sorbitol, 1.5 % mannitol pH 7.5
F7	10 mM histidine, 10 % trehalose, 0.01 % PS-20 pH 6.5
F8	10 mM histidine, 10 % trehalose, 0.01 % PS-20 pH 7.0
F9	10 mM histidine, 10 % trehalose, 0.01 % PS-20 pH 7.5

Study Design

Each of the nine formulations tested in this study are shown in Table 1. Study samples were prepared from a representative sample from a commercial batch of Soliris®(eculizumab) UFDF product, not containing polysorbate 80. Study samples were prepared by dialysis, followed by concentration using concentration devices, as required, under OD280 control. The prepared nine compositions were spiked with 10 w/v % PS-80 or 10 w/v % PS-20 stock solutions as required. Further study sample preparation was conducted in biosafety cabinet to enable sterility of the material in the glass vials during the accelerated

stability testing. Soliris® stocks were 0.22 mm sterile-filtered, filled at 0.5 mL fill into 2mL Schott type I borosilicate glass vials, stoppered with West Pharmaceuticals 4432/50 fluorotech stoppers and crimped. Study samples were incubated at 23-27 °C for up to 12 weeks with testing at 0 weeks, 4 weeks, 8 weeks and 12 weeks.

Two stability-indicating assays were performed: SEC-HPLC and icIEF. SEC-HPLC was used to assess the purity and physical stability of eculizumab in the formulation, and icIEF was used to assess the chemical stability of eculizumab in the formulation and whether it underwent oxidation and/or deamidation reactions.

J A • K E M P

BY ONLINE FILING

The European Patent Office
Bayerstrasse 34
(entrance via Zollstrasse 3)
80335 Munich
Germany

5 July 2019

Dear Sirs

European Patent Application No. 16172335.8
ALEXION PHARMACEUTICALS, INC.
Our Ref: N411599EP-A PXT/ncb

Thank you for the summons to attend oral proceedings dated 27 March 2019. The Applicant wishes to make the following submissions in advance of the hearing scheduled for 5 September 2019.

A new main request and two auxiliary requests are attached. We ask that the Examining Division consider the auxiliary requests in the event that the main request is considered unallowable.

Tracked-changes versions of the current claims showing the amendments being made to derive the claims of the main request and of the main request showing the amendments being made to derive the two auxiliary requests are attached.

I attach a full list of the documents cited in these examination proceedings. A copy of each of D24 to D30 and D32 to D36 is attached.

Main request

In the main request, claim 7 has been amended to depend only on claims 1 to 5.

The claims

Claim 1 of the present application is directed to:

> *"A pharmaceutical composition comprising an antibody that binds C5 in a 300mg single unit dosage form comprising 30 ml of a 10 mg/ml sterile, preservative free solution, wherein the antibody comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of*

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A list of our partners is available at our principal place of business at the address above. Regulated by IPREG

> *SEQ ID NO: 4, for use in treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH).”*

The present application discloses that SEQ ID NO: 2 is the full (variable and constant) heavy chain amino acid sequence of eculizumab, and that SEQ ID NO: 4 is the full (variable and constant) light chain amino acid sequence of eculizumab. This is confirmed in paragraph 6 of the Boone declaration (D13). SEQ ID NO: 2 is the mature sequence and does not include the N-terminal signal peptide. The heavy chain constant region of SEQ ID NO: 2 is a unique, engineered heavy chain constant region. SEQ ID NO: 4 is an immature sequence that includes the 22 amino acid N-terminal signal peptide, which is cleaved off during antibody production in a cell. The light chain constant region of SEQ ID NO: 4 is a kappa constant region.

The claims are therefore directed to a defined composition comprising the specific antibody, eculizumab, which obtained marketing approval under the trade name SOLIRIS® after the filing date of the present application (see D15/D16).

The structure of eculizumab was not disclosed in the prior art

The full heavy chain and light chain amino acid sequences of SOLIRIS® (eculizumab) were first disclosed in the present application. Prior to the filing date of the present application, it was not known that “eculizumab” was an antibody comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 SEQ ID NO: 4.

D1-D5 do not disclose the structure of the claimed antibody

None of D1-D5 includes any structural information for “eculizumab”.

D1 refers to “eculizumab” as being the tradename of the antibody “*h5G1.1-mAb*”. D1 does not include any sequence information, but states that:

> *“Methods for the preparation of h5G1.1-mAb, h5Gl.l-scFv and other functional fragments of h5G1.1 are described in U.S. Pat. No. 6,355,245 and “Inhibition of Complement Activity by Humanized Anti-C5 Antibody and Single Chain Fv”, Thomas et al., Molecular Immunology, Vol. 33, No. 17/18, pages 1389-1401,1996, the disclosures of which are incorporated herein in their entirety by this reference. The antibody h5G1.1-mAb is currently undergoing clinical trials under the tradename eculizumab.”* (D1, page 5, left column last two lines to right column line 8).

U.S. Pat. No. 6,355,245 is D11, and Thomas et al., Molecular Immunology, Vol. 33, No. 17/18, pages 1389-1401,1996 is D7 in the present proceedings.

D2 describes “eculizumab” as “*a humanized monoclonal antibody that binds C5 and inhibits terminal complement activation*” (D2, lines 4-5). No structural information is provided in D2, and it does not reference any other documents.

D3 describes “eculizumab” as “*a recombinant humanized monoclonal antibody that was designed to block the activation of terminal complement components*[14,15]” (D3, left column page 553, third paragraph, first three lines). Reference 14 in D3 is a

general publication relating to humanization of antibodies (Riechmann et al. (1988) "Reshaping human antibodies for therapy" Nature 332: 323-7), and reference 15 is D7 in the present proceedings. No structural information is provided in D3 itself.

D4 describes "eculizumab" as "*a recombinant, fully humanized monoclonal antibody that inhibits the activation of terminal complement components. Eculizumab binds specifically to the terminal complement protein C5 and prevents the release of the inflammatory mediator C5a and the formation of C5b-9 by inhibiting the cleavage of human complement C5 to C5a and C5b (12)*" (D4, page 673, right column, third paragraph, first three lines). Reference 12 in D4 is D7 in the present proceedings. D4 also includes the CAS STN database number "CAS: 219685-50-4" on page 673. However, as discussed below, this database did not include the correct sequence for eculizumab at the filing date of the present application. No structural information is provided in D4 itself.

D5 does not mention "eculizumab".

Thus, D1 to D5 do not provide any sequences for "eculizumab", and only point to D7 and/or D11 as describing "eculizumab".

Neither D7 nor D11 discloses the full structure of the claimed antibody

D11 and D7 are referred to in the cited prior art documents as describing "eculizumab". However, neither D7 nor D11 discloses the amino acid sequences of SOLIRIS® (eculizumab) recited in the present claims.

D7 and D11 describe the cloning of the murine 5G1.1 antibody and the generation of humanised variable region sequences.

The only disclosure relating to full length antibodies in D11 is a general paragraph at column 45, lines 24-33 which describes in general terms how matched pairs of the variable regions (e.g., a VL and a VH region) of the various described antibody molecules, Fds and light chains may be combined with constant region domains to form full length antibodies of the invention.

D7 discloses CDR grafted V_L and V_H sequences (h5G1.1VCH and h5G1.1VLC in Figure 1 of D7) and also V_L and V_H sequences comprising back mutations (h5G1.1VHC+F and h5G1.1VLC+F in Figure 1 of D7) and reports that an intact humanised antibody of the IgG4 isotype was constructed and produced (see page 1396, paragraph spanning columns of D7).

As discussed above, the present claims are directed to an antibody comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4, which are the sequences of the antibody in SOLIRIS® (eculizumab). This claimed antibody is **not** the IgG4 antibody described in D7. In particular, eculizumab does not have an IgG4 heavy chain constant region, but a unique, engineered, non-naturally occurring constant region sequence that has 14 different amino acid residues compared to the IgG4 sequence (4 in the CH1 domain, 6 in the hinge region and 4 in the CH2 domain - see paragraphs 4 and 5 of the Bell declaration (D31)).

Therefore, although D1 refers to D7 and D11, and D3 and D4 refer to D7, supposedly as describing "eculizumab", neither D7 nor D11 discloses the full heavy chain sequence shown in SEQ ID NO: 2 of the present application and the full light chain sequence shown in SEQ ID NO: 4 of the present application.

None of D1-D4, D7 and D11 provide an enabling disclosure of the claimed antibody

In view of the above, the skilled person would not be able to reproduce "eculizumab" based on the disclosures of any of D1-D5, D7 and D11. According to the established case law, a disclosure in a prior art document is novelty-destroying only if the teaching it contains is reproducible (see, for example, T 1437/07, Reason 25, and Guidelines for Examination G-IV-2).

Hence, none of D1-D5 can be considered to belong to the state of the art according to Article 54(2) EPC, at least in as far as they relate to "eculizumab", and consequently none of D1-D5 prejudices the patentability of the claimed invention.

None of the patents listed in D8 provide an enabling disclosure of the claimed antibody

D8 lists a number of Alexion patents and patent applications that refer to "eculizumab". None of the patents and patent applications listed in D8 provide any sequence information for "eculizumab". Insofar as these patents and patent applications use the term "eculizumab", they refer back to D7 and/or D11 as shown in the attached table (D24).

As discussed above, neither D7 nor D11 disclose an anti-C5 antibody as presently recited in the claims, with a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4. These full sequences of the antibody in SOLIRIS® (eculizumab) were not known in the art at the filing date of the present application. Therefore, the prior art disclosures in the patents and patent applications do not enable the skilled person to make the specific antibody that is now recited in the claims.

In this regard, it should be noted that the patent and patent applications listed in D8 are not focused only on eculizumab. These patents and applications relate to complement inhibitors more broadly, including anti-C5 antibodies generally. As shown in D8, with one exception (EP 1720571, the patent granted from D10, which has been revoked (see below)), "eculizumab" is referred to only in dependent claims. Therefore, the fact that there is no enabling disclosure of the specific antibody "eculizumab" does not render all of these patents and patent applications insufficient across their whole scope. The insufficiency extends only to the specific dependent claims that mention "eculizumab".

Comments made by Alexion in D9 in the course of opposition proceedings regarding a different patent do not change what was in fact disclosed in the prior art

The statement made in 2013 by the applicant in the opposition proceedings against the patent derived from D10 does not change what was disclosed in D10 in 2005, and

does not retroactively make the disclosure of D10 , or any other prior art document referring to "eculizumab", enabling in so far as it relates to "eculizumab".

The issue of sufficiency of disclosure of "eculizumab" was raised in the opposition proceedings against EP 1720571 (the patent derived from D10). The Opponent observed that:

> *"Eculizumab is an essential feature of claims 1 to 8. The structure of Eculizumab is not disclosed in the application as filed. On page 8, lines 8-9, the patent discloses that h5G1.1-mAb is currently undergoing clinical trials under the tradename Eculizumab. On the same page, lines 5 to 8, the patent further mentions that methods for the preparation of h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1 are described in U.S. Patent No. 6,355,245 (D9)* [D11 in the present proceedings] *and 'Inhibition of Complement Activity by Humanized Anti-C5 Antibody and Single Chain Fv,' Thomas et al., Molecular Immunology, Vol. 33, No. 17/18, pages 1389-1401, 1996 (D10)* [D7 in the present proceedings].
>
> ***However, as detailed below, the complete sequence of Eculizumab or h5G1.1 mAb is not disclosed in these two documents.***
>
> *D9* [D11] *is a patent assigned to Alexion Pharmaceuticals, Inc. which relates to C-5 specific antibodies for the treatment of inflammatory diseases.*
>
> *In example 7, D9* [D11] *discloses a mouse monoclonal antibody 5G1.1. In example 11 (columns 42 to 44), D9* [D11] *lists a number of humanized, chimeric and non humanized ScFv, Fd and light chains. D9* [D11] *does not comprise any reference to Eculizumab or h5G1.1 mAb nor does it disclose the sequence of a full length humanized antibody.*
>
> *D10* [D7] *is a publication from Alexion Pharmaceuticals relating to humanized anti-C5 antibody and scFv. D10* [D7] *discloses the cloning of the variable region genes of the mouse mAb 5G1.1 (see page 1380), the humanization of 5G1.1 variable regions and the construction and expression of various Fab and scFv (see page 1391). The sequences of humanized variable regions are provided in Figure 1. The construction of a humanized antibody 5G1.1 called h5G1.1 HuG4 is also discussed on page 1396 (paragraph bridging col. 1 and 2) and on page 1399, col.2. However, the exact sequence of the humanized antibody is not disclosed in D10* [D7].
>
> *In view of the above, none of the patent, D9* [D11] *or D10* [D7] *provides a detailed description of the structure of h5G1.1 mAb or Eculizumab.*
>
> *Therefore, the man skilled in the art would not be able to carry out the invention as he would not be able to prepare the specific antibody Eculizumab, which is an essential feature of the claims 1 to 8."* (See section 7.1 of D21)

In defence of its patent, Alexion replied to this attack by asserting that the sequence for eculizumab was publicly available and stated (in D9) that:

> "A *sequence for eculizumab was submitted to Chemical Abstracts Service (CAS) and entered into their STN database on 14 February 1999; please see the attached copy of the CAS entry no. 219685-560-4."*

The copy of CAS entry no. 219685-560-4 provided did not include any sequences, the identity of the sequences made available on 14 February 1999 was not investigated at that time, and this argument was not challenged.

In the preliminary opinion that accompanied the summons to oral proceedings dated 14 January 2014 (D25), the Opposition Division expressed the opinion that:

> "I*n the absence of evidence that* eculizumab *could be identified as a specific antibody, either by deposit or* ***full*** *sequence of its light and heavy chains, the antibody and thus the subject-matter of claims 1-8 cannot be considered to be sufficiently disclosed" (paragraph 2.29 of D25).*

The position of the Opposition Division was not further challenged by Alexion or discussed at the oral proceedings (see Alexion letter of 17 March 2014 (D26), which does not include any arguments relating to sufficiency of disclosure), and EP1720571 was revoked for lack of inventive step (see the Decision of the Opposition Division dated 24 June 2014 (D27)).

The statement made in defence of EP1720571 should have no bearing on the current proceedings. It does not change the facts of what was disclosed prior to the filing date of the present application. Nor did Alexion gain any advantage from the statement made in connection with the opposition to that earlier patent.

In accordance with the opinion of the Opponent and Opposition Division in the opposition against EP1720571 that this patent does not provide a sufficient disclosure of eculizumab, the application for EP1720571, D10, also does not provide an enabling disclosure of eculizumab.

The sequence of eculizumab was not available in the CAS database at the filing date

Heavy and light chain amino acid sequences were submitted to the CAS Registry in 1999 under the name "eculizumab", but, as evidenced by the Saxe Declaration, the sequences submitted to CAS at this time were **not** the sequences of the antibody (eculizumab) in SOLIRIS® to which the present claims are directed. The correct sequences for eculizumab were later submitted to replace the incorrect sequences in May 2009. These facts are not disputed.

However, one argument put forward by the Examining Division is that the availability of incorrect sequences for eculizumab on the CAS Registry database amounts to a public disclosure of the correct sequences for eculizumab because it was the intention of Alexion to make the sequence of eculizumab public at the time, such that Alexion would be obliged to provide the correct sequence of eculizumab to anybody who requested it. The Examining Division has referred to "*CAS rules*" as obliging Alexion to provide such information. However, the Examining Division has not provided evidence that any such rules existed, and Alexion is not aware of any.

The fact therefore remains that the correct sequence of eculizumab was not made available in the CAS Registry prior to the filing date of the present application.

The statement by the applicant in D9 ("A *sequence for eculizumab was submitted to Chemical Abstracts Service (CAS) and entered into their STN database on 14 February 1999; please see the attached copy of the CAS entry no. 219685-560-4.")* does not change the fact that the wrong sequence was included in the CAS database in February 1999 and the fact that this incorrect sequence was still the sequence listed for "eculizumab" in the CAS database at the filing date of the present application. The context of the statement in D9 is explained above. Rather than speculate as to the intent of Alexion in 1999, the proper inquiry should be what in fact was disclosed at the filing date of the present application and whether a person skilled in the art would have known or been able to determine the structure of eculizumab, as recited in the present claims.

The Boards of Appeal have considered the prior art effect of documents that contain mistakes. In particular, as stated in the Case Law of the Boards of Appeal, 8th edition, I-C-4.9, in T 412/91 the board held that:

> *"in principle, what constituted the disclosure of a prior art document was governed not merely by the words actually used in its disclosure, but also by what the publication revealed to the skilled person as a matter of technical reality. If a statement was plainly wrong, whether because of its inherent improbability or because other material showed that it was wrong, then - although published - it did not form part of the state of the art. Conversely, if the skilled person could not see the statement was wrong, then it did form part of the prior art."*

Similarly, in the Case Law of the Boards of Appeal, 8th edition, I-C-4.9, it is noted that:

> *"In T 230/01 the board noted that a document normally forms part of the state of the art, even if its disclosure is deficient, unless it can unequivocally be proven that the disclosure of the document is not enabling, or that the literal disclosure of the document is manifestly erroneous and does not represent the intended technical reality. Such a non-enabling or erroneous disclosure should then not be considered part of the state of the art."*

Applying this reasoning to the present case, either the skilled person would recognise that the sequences of eculizumab in the CAS database must be wrong, in which case the incorrect information is not part of the state of the art, or the sequences in the CAS database do not destroy the novelty of the present claims.

The Board in T 591/90 considered that it could be assumed that a skilled person would try to correct recognisable errors in a prior document if, using his general technical knowledge and consulting the reference literature, he could see at once that the information in question was not correct. (See Case Law of the Boards of Appeal, 8th edition, I-C-4.9.)

In the present case, the skilled person would be unable to correct the errors in the eculizumab sequences in the CAS database based on his common general knowledge and by consulting the literature.

The Saxe declaration (D17) explains that the heavy chain sequence submitted to CAS was much longer (650 amino acids) than the actual heavy chain sequence (448 amino acids). The annotated copy of the sequences originally submitted to CAS attached as D28 (obtained by searching for CAS Registry Number: 219685-50-4 on http://commonchemistry.org/ and annotated by hand) shows how extensive the errors are. In particular, the heavy chain sequence originally submitted to CAS contains three stretches of additional amino acids, has two single amino acid substitutions and is missing a stretch of 16 amino acids compared to the correct sequence. For comparison, an annotated copy of the corrected sequences for CAS Registry Number: 219685-50-4 is attached as D28a.

As discussed above, the skilled person would not have known that eculizumab has a uniquely-engineered heavy chain, and so the skilled person would not have known what the correct heavy chain constant region sequence of eculizumab should be.

Also, the light chain submitted to CAS in 1999 contains a single amino acid substitution compared to the correct sequence in the variable region (see D28 and D28a). The light chain variable region sequence shown in D7 is the same as the incorrect sequence at this position, and so the skilled person would have had no way of knowing that there was an error in the light chain variable region sequence.

Accordingly, the errors in the eculizumab sequences are not errors that would have been recognisable, or at least correctable, by a person skilled in the art at the filing date.

It is worth noting how the EPO would have treated an earlier patent application in which the Applicant had erroneously included the incorrect sequence for eculizumab, such as that submitted to the CAS Registry in 1999. In such a situation, it would have been the intention of the Applicant to disclose the sequence of eculizumab to the public in the patent application. However, the Applicant would not have been allowed to correct the sequence merely because it was his intention to make the correct sequence public and because the correct sequence was necessary to claim eculizumab and meet the sufficiency requirements of Article 83 EPC. To the contrary, the sequence in such a patent application could later be corrected only if it was immediately obvious that the sequence contained an error and also what the correct sequence should be (Rule 139 EPC). In other words, the Applicant's intent would not have been taken into consideration. We refer in this regard to Board of Appeal decision T1111/02 in which there were errors in a sequence provided in the patent application that led to a finding of insufficiency because the incorrect sequences could not be corrected under Rule 88 EPC (now Rule 139 EPC). Accordingly, it is the disclosure itself – rather than the intent of the Applicant – that is controlling. The same should apply in the present case (regardless of what the intent of the Applicant was in 1999).

The fact remains that the CAS database did not contain SEQ ID NOs: 2 and 4 of the present application at the filing date, and the incorrect sequences for eculizumab

submitted to CAS in 1999 did not enable the skilled person to make the antibody defined in the present claims (SOLIRIS® (eculizumab)).

Eculizumab itself was not publicly available at the priority date

Eculizumab used in the clinical trials was not available to the public

The clinical trials described in the prior art did not make the antibody eculizumab available to the public. The position taken by the Examining Division in this regard is inconsistent with established jurisprudence of the Boards of Appeal.

We are filing herewith a new Declaration of Michael Bombara (D36) who managed many of the clinical trials that Alexion conducted in the late 1990s and early 2000s, and whose responsibilities included ensuring that all of the necessary contacts between Alexion and the principle investigators and/or their institutions or study sites were in place (see paragraph 2 of D36). Bombara confirms that no member of the public should have been able to obtain Alexion's confidential information and/or study drug material (eculizumab) in the course of any of the clinical studies (see paragraph 17 of D36).

The Bombara declaration summarises Alexion's clinical trial practice prior to the present filing date and explains the multiple layers of confidentiality that were built into each clinical study to ensure that the principal investigators and study institutions or sites clearly understood their obligation to maintain Alexion's information, including the study drug, in strict confidence (see paragraph 5 of D36).

Bombara refers to one specific example of the agreements in place for the pilot PNH study of 11 PNH patients referenced in D1 to D5 (the "C02-001" study) and provides evidence of the agreements in place for this study. The declaration confirms that the authors on each of D1-D3 and D5 were affiliated with Alexion or one of the two institutions bound by these agreements (see paragraph 15 of D36). Bombara also states that similar documents would have been executed for all of the other eculizumab-related studies conducted by Alexion in the late 1990s and early 2000s (see paragraph 16 of D36).

The Bombara declaration additionally explains that the investigators and institutions agreed to adhere to strict requirements set forth in the clinical trial protocol regarding the tracking, logging, storage and disposal of study drug material, and provides an example of a drug disposal form (see paragraphs 12 and 13 of D36).

As an initial point, the Examining Division is incorrect that "*none of the documents cited in respect of the present application relate to clinical trials*". All of D1 to D5 relate to clinical trials carried out under the control of Alexion:

- D2 and D3 describe a pilot study that was conducted in 11 PNH patients for 12 weeks. This study is the "C02-001" study as identified in the table in the Boone declaration (D13) and exhibit B of the Bombara declaration (D36).
- D5 describes an extension of the "C02-001" study during which the PNH patients were treated for 52 weeks. This extension study is the "E02-001" study identified in the table in the Boone declaration (D13) and exhibit B of the Bombara declaration (D36).

- D1 describes a further extension of the "C02-001" study during which the PNH patients were treated for two years. This extension study is the "X03-001" study identified in the table in the Boone declaration (D13) and exhibit B of the Bombara declaration (D36).
- D4, which discloses Alexion Pharmaceuticals, Inc. as its source (page 675, right column) is a review paper that describes a series of clinical trials that were conducted with eculizumab. All of these trials are studies identified in the table in the Boone declaration (D13) and exhibit B of the Bombara declaration (D36):
    - The study in 117 patients with idiopathic membranous nephropathy is "C99-004".
    - The study in 40 rheumatoid arthritis patients is "C97-001".
    - The study in 209 rheumatoid arthritis patients is "C99-001".
    - The study in 368 rheumatoid arthritis patients is "C001-004".
    - The two month trial in 10 patients with dermatomyositis is "C99-006".
    - The phase II trial in membranous nephritis is "C99-004".
    - The trial in systemic lupus erythematosus is "C97-002".
    - The pilot and extension studies in 11 PNH patients are "C02-001" and "E02-001".

The Boone declaration (D13) explains that all of these trials were carried out under confidentiality agreements and that the eculizumab drug supplies used in the studies were tightly controlled. We have also provided direct evidence of the relevant provisions in the "C02-002" and "C04-001" studies, by way of example. As is clear from paragraphs 7 to 9 of the Boone declaration (D13) and paragraphs 5 and 16 of the Bombara declaration (D36), all of the studies listed in the table included substantively identical confidentiality provisions. It is also stated in paragraph 2 of the Bell declaration (D31) and paragraph 3 of the Bombara declaration (D36) that eculizumab was used in clinical trials before 15 March 2007 only under strict control and conditions of confidentiality.

The Examining Division is asking that we provide proof of a confidentiality agreement between Alexion and each participant/physician involved in the clinical studies. This level of proof is not, however necessary.

It is stated in Case Law of the Boards of Appeal III-G-5.1.1 that:

> "*In ex parte proceedings the applicant bears the burden of proof for the facts in his favour, e.g. the fact that a document cited by the examining division does not form part of the state of the art (**T 160/92**, OJ 1995, 35)*",

However, this statement does not require that the Applicant provide absolute proof. The Case Law of the Boards of Appeal III-G-5.1.1 goes on, in the following paragraph to say:

> "*As far as issues relating to patentability requirements are concerned, the burden of proof in **examination proceedings** cannot lie initially with the applicant. If an examining division raises an objection, it must be appropriately substantiated.* In **T 578/06** the *examining division had failed to provide such substantiation. Also, the passage in the "Case Law of the Boards of Appeal of the EPO" (see paragraph immediately above) could not support*

> *its view that in ex parte proceedings the applicant has the burden of proof for facts in his favour, as it appeared to have been taken out of its context. In this respect, the board clarified that all the procedural situations referred to in this passage were those where, in response to a substantiated objection from the examining division, the applicant was required to support its contention."*

Further, same section of the Case Law of the Boards of Appeal (III-G-5.1.1) goes on to explain that:

> *"Where a lack of novelty is alleged, the burden of proof invariably lies with the party claiming that the information in question was made available to the public before the relevant date (see, for example **T 193/84**, **T 73/86**, **T 162/87**, **T 293/87**, **T 381/87**, OJ 1990, 213; **T 245/88** and **T 82/90**). According to the boards' established case law, each of the parties to the proceedings bears the burden of proof for the facts it alleges. If a party, whose arguments rest on these alleged facts, does not discharge its burden of proof, this is to the detriment of that party, who may not shift the onus of proof onto the other party (see **T 270/90**, OJ 1993, 725; **T 355/97**; **T 836/02**; **T 176/04**; **T 175/09**; **T 443/09**)."*

We have provided direct evidence of confidentiality agreements in some of the studies using eculizumab (D22, D23 and D36), and declaratory evidence that confidentiality provisions applied to all of the studies using eculizumab (D13, D31 and D36). The Examining Division, on the other hand, has not substantiated its arguments that eculizumab was made available to the public as a result of the clinical trials carried out before the filing date, nor has the Examining Division provided any contrary evidence to that we have submitted.

The evidence we have provided does not include any confidentiality agreements with the patients taking part in the clinical trials. However, the patients were never in a position to inspect or distribute eculizumab. Eculizumab is administered by intravenous infusion in the clinic (see Bell declaration (D31), paragraph 2 and Bombara declaration (D36), paragraph 3). Therefore, the intravenous administration of eculizumab is akin to the implantation of a prototype device (T152/03 and T906/01) and the eculizumab clinical trials can be distinguished from the trials in T7/07 where a large number of patients were given tablets to take home with them for use over a longer period of time (see T7/07 Reason 3.3). The eculizumab used in the trials carried out prior to the filing date of the present application remained under the strict control of health care practitioners who were bound by strict confidentiality obligations and was not made available or accessible to the public.

Further, it is established in T152/03 and T906/01 that the development and test phases of implanted products were necessarily surrounded by secrecy as long as such products had not been commercialised, and that there was a prima facie assumption that any person involved in a medical tests of such products was obliged to maintain confidentiality and that any evidence proving the contrary was important. In the present case, eculizumab had not been commercialised at the filing date (see D13 paragraph 14, D15 and D16. There is therefore an assumption that those involved with the trials were obliged to maintain confidentiality. Furthermore, we have provided evidence that the trials were carried out under confidentiality agreements

with Alexion and that eculizumab used in the clinical trials remained under the strict control of Alexion (see D13, D31 and D36).

As stated in the Case Law of the Boards of Appeal, 8th edition, III-G-4.3.1:

> *"The EPO departments decide on the issues that arise before them on the basis of the evidence adduced by the parties. Their decisions need not, and in most cases cannot, be based on absolute conviction, but instead are to be arrived at on the basis of the overall balance of probabilities, in other words on the footing that one set of facts is more likely to be true than the other. If the result of the boards' evaluation of the evidence is such as to persuade them one way or another, then the* ***balance of probabilities*** *standard is met. This standard applies particularly in opposition appeal proceedings where the boards of appeal are called upon to reach a conclusion on the basis of the overall balance of probabilities, as distinct from "beyond all reasonable doubt" or "absolute conviction" (on the latter concept, refer to the more recent case* ***T 2451/13****). Each of the parties must therefore seek to prove facts alleged by it to that degree of proof (see e.g.* ***T 182/89****, OJ 1991, 391;* ***T 270/90****, OJ 1993, 725;* ***T 859/90****,* ***T 109/91****,* ***T 409/91****, OJ 1994, 653;* ***T 1054/92****,* ***T 296/93****, OJ 1995, 627;* ***T 326/93****,* ***T 343/95****,* ***T 363/96****). The standard of balance of probabilities applies also in decisions issued in ex parte proceedings (****T 381/87****, OJ 1990, 213;* ***T 69/86****,* ***T 128/87****, OJ 1989, 406;* ***T 939/92****, OJ 1996, 309).*
>
> *When an issue of fact is being examined and decided by the EPO on the balance of probabilities,* ***the more serious the issue*** *the more convincing must the evidence be to support it. If a decision on such an issue may result in refusal or revocation of a European patent, for example, in a case concerning alleged prior publication or prior use, the available evidence in relation to that issue must be very critically and strictly examined. A European patent should not be refused or revoked unless the grounds for refusal or revocation (that is, the legal and factual reasons) are fully and properly proved (****T 750/94****, OJ 1998, 32;* ***T 329/02****). See also Guidelines G-IV, 1 "State of the art – General remarks and definition" – November 2015 version): "If the applicant shows sound reasons for doubting whether the document forms part of the 'state of the art' in relation to his application and any further investigation does not produce evidence sufficient to remove that doubt, the examiner should not pursue the matter further."* [emphasis added]

The evidence we have submitted shows that the clinical trials using eculizumab were carried out under confidentiality agreements and that Alexion imposed strict controls on the eculizumab used in the trials. The eculizumab used in the trials was not available to the public. There was no public prior use of eculizumab, and the documents that describe the clinical trials that refer to eculizumab only by name (D1-D5, D10 and the patents and applications listed in D8 that have an earlier filing date that the present application) do not provide an enabling disclosure of eculizumab.

Use of the term "eculizumab" is not evidence that it was publicly available

The Examining Division is incorrect that the fact that many patent and non-patent documents in the prior art related to the use of "eculizumab" is evidence that the compound is well-known and available to the public.

It is established Board of Appeal case law that simply mentioning a biological material by name in a document does not make it publicly available. As stated by the Board in T 576/91, if contractual obligations between parties resulted in access to biological material being deliberately restricted to a group of persons bound either by a research contract or a licence, it could not be concluded that this material had been made "available to the public" within the meaning of Article 54(2) EPC.

In this case, access to eculizumab was limited to the physicians involved in the trials who had signed confidentiality agreements with Alexion, agreed to store eculizumab in a secure, limited-access area and agreed to strict provisions regarding the tracking and disposal of any unused eculizumab. The mention of "eculizumab" by name in public reports of the trials and in patent applications is therefore not evidence that eculizumab was publicly available.

Thus, the absence of a statement in D1-D5 that eculizumab had not been "*available for public use or that the working group using it had been bound to a confidentiality agreement with Alexion Pharmaceuticals"* does not mean that the antibody was available to the public. The evidence shows that the working group using eculizumab was bound to a confidentiality agreement with Alexion Pharmaceuticals. Therefore, in accordance with the case law (T 576/91), it cannot be concluded that eculizumab had been made "available to the public" within the meaning of Article 54(2) EPC.

Articles 76(1) and 123(2) EPC

The amendment to refer to a light chain consisting of residues 23-214 of SEQ ID NO: 4, and to a light chain variable region consisting of residues 23-109 of SEQ ID NO: 3 is an obvious correction that meets with the requirements of Rule 139 EPC.

The Examining Division considers that:

> *"it cannot be excluded by the skilled reader that the SEQ ID NO: 3 and 4 as filed in the parent application and the application as originally filed were actually those for which protection was sought."*

The Examining Division notes that neither the original application nor the parent application contain a disclosure of the fact that SEQ ID NOs: 3 and 4 did not have to include the leader peptide. Such an indication is not, however, necessary because it was part of the skilled person's common general knowledge that antibodies do not include leader peptides. Evidence in support of the skilled person's common general knowledge has already been filed as D14, D19 and D20.

As established by the Enlarged Board of Appeal in G3/89 and G11/91:

> "T*he part of a European patent application or of a European patent relating to the disclosure (the description, claims and drawings) may be corrected under Rule 88, second sentence, EPC [now Rule 139, second sentence, EPC] only within the limit of what a skilled person would derive directly and*

> *unambiguously, using common general knowledge, and seen objectively and relative to the date of filing, from the whole of these documents as filed. Such a correction is of a strictly declaratory nature and thus does not infringe the prohibition of extension under Article 123(2) EPC."* (Head note 1; emphasis added)

The Examining Division talks in terms of protection being sought for SEQ ID NOs: 3 and 4. However, protection of these sequences is not what is sought. Rather, it is protection for an antibody defined in terms of its sequences that is sought by the Applicant. Therefore, it is not correction of SEQ ID NOs: 3 and 4 that is requested. Rather, correction is requested only of the definition of an antibody defined with reference to these sequences.

It would have been immediately apparent to the skilled person that the eculizumab light chain sequences shown in SEQ ID NOs: 3 and 4 of the original application and of the parent application include a 22 amino acid signal peptide at the N-terminus. The skilled person would know from his common general knowledge that the 22 amino acid signal peptide in SEQ ID NOs: 3 and 4 would be cleaved off during antibody production in the cell.

The skilled person would consequently immediately recognise that the recitation at page 5, lines 30-33 of the original application and of the parent application of an antibody that comprises a light chain that "*consists of SEQ ID NO: 4*" is an error, and that the recitation at page 5, lines 25-28 of an antibody having a light chain variable region that "*consists of SEQ ID NO: 3*" is an error.

The skilled person, based on his common general knowledge, would also immediately know that it was the Applicant's intention to define the antibody as including only the mature light chain sequence, i.e. residues 23-214 of SEQ ID NO: 4 or residues 23-109 of SEQ ID NO: 3.

The correction to define the antibody only by reference to the mature light chain sequence is therefore an obvious correction in accordance with Rule 139 EPC, and the present application meets the requirements of Article 76(1) and 123(2) EPC.

In view of the Examiner's comments, claim 7 has been amended to depend only on claims 1 to 5 in order to facilitate prosecution of this application.

The Examining Division has implied that use of the term "*eculizumab*" not the term "*the antibody*" may add subject matter if the antibody in claim 1 and eculizumab do not represent the same entity because the application references D11.
Page 16, lines 24-25 of the present application states that: "*These antibodies disclosed in U.S. Patent 6,355,245 [D11] include a preferred whole antibody (now named eculizumab)*". However, despite the statement made in the present application, D11 does not in fact disclose the sequences of any whole antibody.

As discussed above, the only disclosure relating to full length antibodies in D11 is a general paragraph at column 45, lines 24-33 which describes in general terms how matched pairs of the variable regions (e.g., a VL and a VH region) of the various described antibody molecules, Fds and light chains may be combined with constant region domains to form full length antibodies of the invention.

The statement in the present application is therefore correctly interpreted as meaning that D11 discloses a genus of antibodies that includes the preferred antibody of the present invention (eculizumab), and not that this antibody is explicitly disclosed in D11. It is a matter of fact that D11 does not disclose an antibody with a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4 as presently claimed.

Furthermore, it is confirmed in paragraph 6 of the Boone declaration (D13) that the heavy and light chain sequences of SOLIRIS® (eculizumab) are those in SEQ ID NOs: 2 and 4 of the application. Therefore, the reference in claim 10 to "*the antibody*" does not add subject matter contrary to Articles 76(1) and 123(2) EPC.

Sufficiency - Article 83 EPC

The insufficiency objection raised by the Examining Division arises because the Examining Division does not accept that the correction made to define the antibody in terms of the mature heavy and light chain sequences is an obvious correction of an obvious error. However, the Examining Division has misinterpreted the Applicant's arguments. The application does not include the wrong sequences. SEQ ID NOs: 3 and 4 are the correct light chain variable and full light chain sequences, respectively, but they contain the 22 amino acid signal peptide that is cleaved off during antibody production and so is not present in the mature antibody which comprises two heavy chains and two light chains. Accordingly, the sequences filed in the original application do identify the structure of eculizumab.

It is useful here to briefly set out the legal position in relation to sufficiency of medical use claims. It is well established before the Boards of Appeal that in order to fulfil Article 83 EPC in relation to a medical use claim, (a) the skilled person must be able to produce the composition defined in the claim, and (b) it must be plausible that the composition could be used in the specified treatment. The level of disclosure required in relation to the medical use is set out in Decision T609/02. This describes the now well established principle that the test is a threshold test: all that is required is basic information, either in the patent specification or from the skilled person's common knowledge at the filing date of the patent, which makes the claimed therapeutic effect plausible.

Applying these principles to the present case, in order to fulfil Article 83 EPC, it is necessary that (a) the skilled person could produce a composition comprising 30ml of a 10mg eculizumab per ml sterile preservative free solution, and (b) there must be some indication to make it plausible that the composition would be useful in the treatment of PNH.

Claim 1 defines the antibody by reference to its full mature heavy and light chain sequences. It is routine for the skilled person to make an antibody having known full mature heavy and light chain sequences. Therefore, there is no question that the skilled person would be able to make the antibody of claim 1 without undue burden.

Moreover, the skilled person would have no difficulty in formulating this antibody into a composition having a 10 mg/ml concentration. The skilled person in the art would be familiar with producing compositions comprising antibodies. Further, there

is additional information and guidance provided in the specification at paragraph [0063] onwards. Accordingly, there is no undue burden involved in producing a 10 mg/ml solution of the antibody, based on the information in the specification and the skilled person's common knowledge at the filing date. Point (a) above is therefore fulfilled.

With regard to (b), the application discloses SEQ ID NOs: 2 and 4 as being the heavy and light chain amino acid sequences of eculizumab, and the Examples describe a successful clinical trial using eculizumab to treat PNH in patients. The evidence in the application makes it more than plausible that the claimed composition would be useful in the treatment of PNH. The threshold test defined by T609/02 is therefore met.

Inventive Step - Article 56 EPC

The Examining Division has identified the difference between the "*disclosure of the prior art relating to eculizumab (e.g. D1-D4)*" and the present invention as lying in the sequence of the claimed compound. The Examining Division refers to the incorrect CAS database entry for eculizumab as was available at the filing date. It is argued by the Examination Division that the contribution of the patent is correction of an error in a registered sequence listing, which is devoid of technical character. The Examining Division then asserts that the skilled person had the knowledge to "*carry out the sequence of eculizumab (see the teaching of D7)*". This is not the correct application of the problem and solution approach.

As discussed above, there is no enabling disclosure of "eculizumab" in the prior art. Therefore, the teachings of D1-D4 in so far as they refer to "eculizumab" are not part of the state of the art under Article 54(2) EPC (T1437/05).

The closest prior art

The present application is concerned with providing treatments for PNH. Therefore the closest prior art document is one that is also concerned with treating PNH.

D1 is concerned with treating PNH, and other haemolytic diseases, using inhibitors of complement components, such as complement-inhibiting antibodies. D1 describes a clinical trial using the specific anti-C5 antibody "eculizumab". D1 does not disclose the sequence of eculizumab, but cites D7 and D11 as describing suitable anti-C5 antibodies including "eculizumab". As explained above, D11 does not disclose the full sequence of any antibody and the only full length antibody described in D7 is an IgG4 isotype antibody. Therefore, the teaching in D1 would have led the skilled person to an antibody having a naturally occurring IgG4 heavy chain constant region. Thus, whilst D1 does not provide an enabling disclosure of "eculizumab" for treating PNH, it does provide an enabling disclosure of the IgG4 antibody disclosed in D7 for treating PNH.

The antibody in the composition of claim 1 therefore differs from the antibody in D1 in that the antibody in the claim is not an IgG4 antibody, but has an engineered heavy chain constant region.

D1 also does not disclose a single-use dosage form of a sterile, preservative-free solution comprising 300 mg "eculizumab" at a concentration of 10 mg/ml.

The objective technical problem

The amino acid differences between the IgG4 heavy chain constant region and the engineered heavy chain constant region of the antibody of claim 1 are shown in the Bell declaration (D31) in paragraph 5. The technical effects of these differences are also set out in D31. Paragraph 6 of D31 explains that differences in the heavy chain constant region result in the antibody having reduced binding to Fcγ receptors such that immune responses that are undesirable for an antibody intended to shut down the terminal complement pathway are minimised (see also the experimental report, D18). Paragraph 7 of D31 explains that differences in the heavy chain constant region result in the antibody having an increased plasma half-life (see also the experimental report attached as D29).

The use of the claimed composition of the antibody provides a low volume liquid formulation which is advantageous for IV administration, and which has a very good stability. Using a low volume liquid formulation for IV administration is advantageous. In particular, providing a solution formulation avoids the need for reconstitution prior to IV administration. This reduces the risks associated with incomplete dissolution and avoids inconvenience for clinical staff. Further, the low volume of the composition means it is suitable for administration to a patient within an acceptable infusion time.

Regarding the improved stability of the composition, D32 demonstrates the improved stability, and reduced formation of aggregate, of the 10 mg/ml composition compared with a 30 mg/ml composition.

Figures 1 and 2 of D32 demonstrate that the percentage of eculizumab monomer in the composition decreases over time due to degradation of the antibody. Both the room temperature degradation (Figure 2) and the accelerated degradation (Figure 1, increased temperature) resulted in an approximately linear reduction of monomer content for both compositions studied. The rate of degradation of the 30mg/ml composition is greater than that of the 10 mg/ml composition, under both the storage conditions studied, and throughout the time period studied.

Dr Miano comments in his declaration that after 2 years storage at 2°C to 8°C, the 30 mg/ml composition would have a monomer content which has dropped below the threshold set for product release (below 98% monomer). A similar drop below the 98% monomer threshold occurs under accelerated conditions (at 23°C to 27°C) at six months. However, both Figure 1 and Figure 2 also show that reduction in the percentage monomer is observed not only after 2 years, but from the outset of the experiment. The rate of degradation of the 30 mg/ml eculizumab formulation is greater than that of the 10 mg/ml eculizumab formulation, at all time points. Thus, at any time point after make-up of the composition, the percentage monomer remaining in the composition will be greater for a 10 mg/ml composition than for a 30 mg/ml composition.

The data of D32 therefore demonstrate that an eculizumab composition having a concentration of 10 mg/ml will have a greater monomer content, at the point at which it is administered to the patient, than a composition which is identical in all respects except for having a 30 mg/ml concentration. Since from a practical perspective there is always some delay between making up a composition and administering that composition to a patient, the increase in percentage monomer in the 10 mg/ml eculizumab composition is of significant benefit to the patient, since it ensures a greater amount of the active substance is administered.

Aggregation is well known to be disadvantageous in the context of a therapeutic antibody treatment. In particular, aggregation can lead to immunogenicity and loss of efficacy. To demonstrate the effects of eculizumab aggregation, a second declaration of Dr Miano, D33, describes the properties of the impurity produced on storage of an eculizumab composition. In the experiments described in D33, the impurity was isolated and studied by SEC (see Figure 1) and it was confirmed that a dimer of eculizumab had formed.

C5 binding activity studies showed that the activity of the dimer is approximately 50% of that of eculizumab itself. Thus, D33 demonstrates that a significant drop in potency occurs on aggregation of eculizumab to the dimer form.

The data of D32 and D33 collectively show that the delay between making up a composition of eculizumab, and administering that composition to a patient, can have a greater impact on the resulting treatment in the case of a 30 mg/ml composition, compared with a 10 mg/ml composition. The 10 mg/ml composition will retain a higher proportion of the intact monomer, and will accordingly retain a higher activity. Thus, the technical effect of the 10 mg/ml composition is to provide a highly stable composition of eculizumab, which minimises loss of activity in the period between when the composition is produced and when the composition is administered to the patient.

D34 is a third declaration of Dr Dino Miano, in which stability studies are described of nine different eculizumab compositions. These compositions include three different sets of formulation excipients, and three different pH levels. Composition F2 corresponds to SOLIRIS®, the formulation tested in D32 and D33. F1 and F3 are identical compositions except for variation in the pH. The formulation excipients in compositions F4 to F9 differ from those used in F1 to F3. For instance, F4 to F6 do not contain any surfactant. Further, F4 to F9 use a histidine buffer rather than a phosphate buffer, and each of F2, F5 and F8 vary in terms of their osmolality (ionic strength).

For each of the nine different compositions, Dr Miano studied the formation of aggregates in both a 10 mg/ml composition and a 30 mg/ml composition. These experiments were carried out in the same temperature controlled unit and thus each composition was subject to identical conditions. In each case, Dr Miano found the 10 mg/ml composition to be more stable to aggregation than the corresponding 30 mg/ml composition having the same formulation and pH.

D34 therefore demonstrates that the objective technical problem (providing an antibody, in a composition which is highly stable, and which can be provided in a low volume liquid formulation) is solved by use of a 10 mg/ml composition for a wide

range of different compositions, including those having varying pH and formulation excipients. D34 thus provides evidence that the technical problem is solved across the full scope of the claim.

The objective technical problem in view of D1 which is solved by the invention is therefore the development of an antibody having improved properties compared to the IgG4 antibody of D1/D7 for use in the treatment of PNH, in a composition which is highly stable, and which can be provided in a low volume liquid formulation.

The solution is not obvious

The solution to this problem provided by the claimed composition would not have been obvious to the skilled person at the filing date.

Firstly, the skilled person would not have been motivated to find an alternative to the IgG4 antibody of D7 referred to in D1.

D7 explains why the IgG4 isotype was chosen for the full length antibody, reporting that:

> *"The human IgG4 isotype was chosen, as this isotype does not activate human complement (Tao et al., 1993) and there is only one known allotype of IgG4 (Ghanem et al., 1988), precluding the potential development of allo-antibodies in patients. The humanized h5G1.1 (CDR) HuG4 antibody bound to human C5 with a similar avidity as the murine antibody when assayed by ELISA (Fig. 9) and inhibited lysis of porcine aortic endothelial cells as effectively as the murine antibody, with a 1:1 molar ratio of antibody binding sites to human C5 being sufficient for inhibition (Fig. 10)" (D13, page 1399, right column, second paragraph, lines 15-25)."*

The skilled person would have had no motivation to use an antibody different than the IgG4 antibody described in D1 with reference to D7 in view of the report in D1 of the successful treatment of PNH, and the teaching in D7 discussed above that the human IgG4 isotype has the advantages that it does not activate human complement and has only one known allotype, precluding an immune response against the constant region of the antibody.

In particular, the skilled person would not consider using a different, engineered constant region sequence in place of the IgG4 isotype antibody disclosed in D7 because he would be concerned about the immunogenicity of the therapeutic antibody, and D7 teaches that the IgG4 isotype is ideal in this regard. The safety of the IgG4 antibody of D7 would have been confirmed to a skilled person by the clinical studies reporting that "eculizumab" (identified as the antibody of D7) was safe and effective when administered to PNH patients (e.g. D1, D3 and D4). Also, at the filing date of the application, other IgG4 isotype antibodies had been commercialised as therapeutic antibody products (see D30, table 1). Significantly, at the filing date of the application, no antibody having the uniquely-engineered heavy chain constant region of SEQ ID NO: 2 was on the market or publicly known to be studied in human clinical trials.

Secondly, the advantageous properties of reduced immunogenicity and increased half-life exhibited by the claimed antibody are not taught or suggested in any of the cited documents. Indeed, in paragraph 8 of D31, it is explained that the advantageous properties of the claimed antibody over the antibody of D7 (which is the antibody to which D1 relates) were surprising and unpredictable.

Accordingly, claim 1 is inventive because it relates to a novel antibody with unexpected advantageous properties.

Furthermore, the composition comprising an antibody as defined in the claims as a single unit dosage form comprising 30ml of a 10mg/ml sterile, preservative free solution for treating PNH is also inventive.

There is nothing in the prior art which would suggest to the skilled person that a 10 mg/ml composition would be beneficial, let alone that this composition would solve the above defined objective technical problem. The Examining Division has referred to D12 as describing formulations of "eculizumab" at concentrations of between 1 and 30mg/ml and has noted that in the absence of any special technical effect of the claimed composition and any prejudice in the art to the formulation of eculizumab at the specific concentration claimed, D12 would have led the skilled person to the 10mg/ml composition now claimed.

However, D12 does not provide an enabling disclosure of "eculizumab" for the same reasons as discussed above in relation to other prior art documents. D12 discloses that examples of useful antibodies "*include anti-C5 antibody or antibodies that inhibit activation of the complement cascade, for example the antibodies described in U.S. Pat. No. 6,355,264*", and that "*Certain preferred embodiments employ pexelizumab or eculizumab as the antibody therapeutic*" (paragraph [0004]). The sequence of SEQ ID NOs: 2 and 4 of the present application are not disclosed in D12. Therefore, the disclosure of D12 in relation to "eculizumab" does not form part of the state of the art according to Article 52(2) EPC (T1437/05). Even if "eculizumab" were enabled, D12 would not lead the skilled person to the 10mg/ml composition of claim 1.

D12 is concerned with antibodies which can be used to prevent or treat a pulmonary disease or condition (see abstract and paragraph [0011]). D12 is not concerned with treating PNH.

Further, D12 is specifically concerned with formulating antibodies for administration directly to the lung, by inhalation. This may be by inhalation of an aerosolised composition (paragraph [0006]), or by nebulisation (paragraph [0007]). The only mention of a formulation containing "eculizumab" is under the heading "Nebulisation Formulations" at paragraph [0169]. There is no mention of systemic administration of "eculizumab" in D12. In fact, paragraph [0004] (see the passage spanning the two columns of page 1) indicates that the methods may specifically be for administration other than systemically. In contrast, treatment of PNH requires compositions for IV administration.

Paragraph [0169] of D12 onwards refers to a specific formulation which is in accordance with Formulation 1 and has an "eculizumab" content of 30 mg/ml (paragraph [0171]). Notably, however, Formulation 1 is not described as a solution.

As is well known in the art, and discussed for instance at paragraph [0074] of D12, a formulation for nebulisation may be a solution, suspension or slurry. Indeed, budesonide is commonly administered by nebulising a suspension (as noted in paragraph [0062] of D12, with reference to the drug Pulmicort). Therefore, the lack of any mention that the formulation is a solution is significant in the context of a formulation for nebulization. Paragraph [0171] of D12 does not recite a 30 mg/ml "solution". It cannot be assumed that 30 mg/ml formulation is a solution simply because it is intended for nebulization; it could be a slurry or suspension.

There is further reference at paragraph [0173] of D12 to nebulisation of a formulation of "eculizumab." SDS-PAGE and HPLC analyses are carried out following nebulisation. However, this paragraph does not indicate the nature of that formulation, merely referring to it as "a suitable formulation."

The present invention is concerned with providing compositions of an antibody which are suitable for treating PNH (which, as is known from D1, is administered systemically via IV). The skilled person would therefore be trying to answer the question of what composition would be suitable for treating PNH, and would thus be interested in formulations for IV administration. More particularly, the skilled person would be concerned with compositions which would solve the objective technical problem, i.e. compositions for use in the treatment of PNH which can be provided as a low volume liquid composition and have good antibody stability. D12 would be taken into account by the skilled person only to the extent that it assists in answering this question.

The indication which is to be treated in D12 is very different from that of the present invention. D12 aims to deliver drug to the lungs to treat pulmonary disease and specifically notes that administration may be non-systemic. In contrast, the present invention aims to treat PNH via a systemic administration. It cannot be assumed that the same composition will be safe and efficacious for both.

There are also fundamental differences in the means of administration described. For instance, during nebulisation, only a proportion of the nebulised material is actually inhaled by the patient. The amount of drug which leaves the nebuliser and is available for inhalation by the patient is indicated by the "nebuliser efficiency". In D12, the nebuliser efficiency ranges from 11.24 to 52.46 % (see Figure 13). Further, of those particles which exit the nebuliser, only a proportion are within the "respirable fraction", i.e. the fraction of drug which reaches the alveoli and accordingly has a therapeutic effect on the patient. D12, Figure 13 also indicates the respirable fraction, which ranges from 44.91 to 74.21%. Thus, only a relatively low amount of the drug which is nebulised actually therapeutically affects the patient. In contrast, administering the same drug via IV will cause 100% of the drug to enter the patient's blood stream.

D12 would not, therefore, lead the skilled person to understand that 1-30 mg/ml solutions of "eculizumab" would be safe and efficacious for treatment of PNH by IV administration.

In fact, the considerations which the skilled person would be concerned with when seeking a suitable composition for IV administration are very different from those

which would be considered for a nebulised formulation. For nebulisation, important factors are the size of the particles produced, since this influences the respirable fraction of the drug, and the ability of the drug to withstand the nebulisation process.

On the other hand, for IV administration, a much more important consideration is whether the drug can be provided in solution form, without particulate matter present. For this reason, there is much incentive to provide a liquid formulation which avoids the risks of incomplete dissolution by a clinician, and which is stable in solution form. Such a requirement is not present for a nebulised formulation, which can be administered as a suspension or slurry. Similarly, the burden on the patient of high volume doses delivered via IV can be problematic and leads to poor patient compliance in connection with repeat treatments. Low volume is therefore also an important factor for an IV administered drug. Again, this factor is not important for nebulisation.

These different considerations lead, in practice, to drugs being formulated in very different ways for the two administration routes. Salbutamol, for instance, is commonly administered via nebuliser at a concentration of 2 mg/ml. In contrast, when this drug is administered via IV, a very different concentration of 0.5 mg/ml or 1 mg/ml is used (see D35, page 3).

The skilled person aware of the D12 disclosure, therefore, would not consider that this helps them to determine whether the "eculizumab" formulations described are safe and efficacious for treating PNH. Compositions for treating different diseases, via different routes of administration, require entirely separate study. For this reason, D12 is not relevant to the question which the skilled person would need to answer in connection with the present invention, i.e. what compositions may be suitable for treating PNH. D12 would not therefore be taken into account by the skilled person.

Moreover, D12 makes no reference to an antibody having the heavy chain shown in SEQ ID NO: 2 and the light chain shown in SEQ ID NO: 4, nor to the ability of the skilled person to provide a composition of that antibody in a highly stable solution at low volume. Regarding the antibody disclosed in D12, D12 refers to "eculizumab" in general terms but does not provide any sequence. It refers to the US patent referred to in the present proceedings as D11 as providing examples of anti-C5 antibodies, but as discussed above D11 does not refer to "eculizumab" and does not disclose SEQ ID NOs: 2 and 4 of the patent (see paragraph [0004]).

Regarding the "eculizumab" compositions, there is no mention of any means to improve the stability of the compositions, and no mention that this can achieved at a concentration of 10 mg/ml. Indeed, the only specific composition exemplified in D12 is that at paragraph [0171] which has a concentration of 30 mg/ml. In this respect, D12 teaches away from the improved compositions now claimed.

Accordingly, D12 does not assist the skilled person in finding a solution to the objective technical problem. Even if the skilled person had considered D12, it would not have led them to produce the claimed composition, in expectation of achieving a high stability, low volume solution of eculizumab.

The claimed subject matter is therefore inventive over D1 combined with D12.

No Double Patenting

The Examining Division has noted that EP 2359834 has been granted with claims to subject matter which is "very similar" to that presently claimed. However, the Examination Guidelines G-IV-5.4 provide that in situations where two applications have the same filing date and relate to the same invention there is no double patenting if one of the applications is amended so that the subject matter of the claims is not identical.

In the present case, the claims of the present application are not identical to the granted claims/main request of EP 2359834. The granted claims of EP 2359834 do not specify any sequence for eculizumab. Therefore the present claims are not identical to those of EP 2359834, and there is no double patenting.

First Auxiliary Request

In the first Auxiliary Request, the antibody is defined as "*comprising the heavy chain amino acid shown in SEQ ID NO: 2 and the light chain amino acid sequence shown in SEQ ID NO: 4*". Basis for this amendment may be found in the originally filed application at page 4, line 32 and page 44.

Second Auxiliary Request

The second Auxiliary Request is the same as the Main Request, except that the subject matter of claim 2 has been incorporated into claim 1.

Conclusion

It is believed that the application is now in allowable form and the Examining Division is requested to reconsider it favourably. The Applicant has addressed each and every issue identified by the Examining Division in the Summons to Oral Proceedings. The Applicant would appreciate it if the Examining Division would consider the response as soon as possible and provide feedback. It is hoped that the application can be allowed without the need for Oral Proceedings on 5 September 2019, on the basis of the Main Request or, failing that, on one of the Auxiliary Requests. Accordingly, the Examiner is requested to telephone the undersigned once the Examining Division has considered our submissions.

I look forward to hearing from the Examiner.

Yours faithfully

Electronically Signed
DR PAMELA M TUXWORTH
ASSOCIATION NO. 811

DECLARATION BY MICHAEL BOMBARA

I, Michael Bombara, Executive Director, Clinical Business Operations – R&D, of Alexion Pharmaceuticals, Inc., of 121 Seaport Boulevard, Boston, MA, United States of America, make the following factual declaration concerning the clinical trial practices at Alexion in the late 1990s and early 2000s when Alexion conducted several clinical studies using eculizumab:

1. I am Executive Director, Clinical Business Operations – R&D, of Alexion Pharmaceuticals, Inc. ("Alexion"), as shown in my curriculum vitae, a copy of which is attached as Exhibit A.

2. I obtained my Bachelor of Science degree in Microbiology in 1984 from the University of Maine. Thereafter, I held laboratory scientist positions of increasing responsibility from 1984 through 1996 at Genentech Incorporated and Bayer Corporation. In 1996 when I became a Medical Research Associate at Bayer, my main work responsibilities began focusing on day-to-day clinical trial management. Two years later I joined Alexion as a Clinical Project Leader. From 1998 through the present I have been working in the Clinical Operations group at Alexion in various roles: from a Clinical Project Leader in 1998 to a Clinical Project Manager in 2001 to now a Clinical Business Operations Lead, which is the role I have held since 2013. I have lead more than a dozen clinical trials at Alexion for several investigative drugs in various indications. Through this work, I have been involved in the day-to-day clinical study-related activities at Alexion, including working with principal investigators and clinical study sites in order to get studies started and make sure they are run in accordance with Alexion's best practices (and also in accordance with all applicable laws and regulations). My responsibilities have included ensuring that all of the necessary contracts between Alexion and the principal investigators and/or their institutions or study sites are in place and making sure that each study protocol is signed by all participating investigators. As I was working as a Clinical Project Manager at Alexion in the late 1990s and early 2000s, I managed many of the clinical trials that Alexion conducted using eculizumab during that time-frame, including a pilot study in which patients afflicted with paroxysmal nocturnal hemoglobinuria ("PNH") were treated with eculizumab, as well as two extension studies thereof.

3. I understand that D1 to D5 are literature references that the European Patent Office has referred to in its "Summons to attend oral proceedings" dated March 27, 2019. I have reviewed each of these documents and explain below how the studies described in each of them were carried out in a manner in which eculizumab and information relating thereto, could not have been disclosed to or used by the public due to: (1) strict confidentiality provisions (in not one but at least two separate agreements governing each study) that each physician and his/her institution involved in the study were bound to abide by; and (2) strict drug accountability provisions in each study protocol that each investigator and institution were bound by, including the requirement that the study drug

eculizumab be stored in only a secure, limited-access storage area. In addition, given that eculizumab has always been administered intravenously, in all of these clinical studies the eculizumab was administered to each patient at a healthcare facility by a healthcare professional who was bound by the clinical study protocol; this is not a case where the patient could have administered the drug to himself, at home, or would have ever had an opportunity to take the drug out of the facility where he was treated. Attached as Exhibit B is also a summary of references D1 to D5 showing the clinical study or studies that each reference describes and whether participants in those studies were bound to keep Alexion's information and drug substance confidential.

4. For many of the studies described in D1 to D5 above, I participated first-hand in ensuring that all of the necessary agreements were in place before a study investigator received any confidential Alexion information and before the clinical study site received any study drug (eculizumab). Alexion was extremely careful in maintaining confidentiality around each ongoing trial and the drug substance used therein (eculizumab).

Confidentiality Regarding Each Clinical Study

5. It was Alexion's clinical trial practice in at least the late 1990s and early 2000s to have multiple layers of confidentiality built in to each clinical study to ensure that the participating investigators and study institutions or sites clearly understood their obligation to maintain Alexion's information, including the study drug, in strict confidence. For example, Alexion typically entered into the following types of agreements for each study:

- Master Clinical Study Agreement ("MCSA"): Alexion entered into this Agreement with an institution or site that it intended to participate with to carry out one or more clinical studies. This Agreement set forth the general terms – including confidentiality requirements – that the parties agreed to abide by in cooperating with one another in the course of performing the study.

- Exhibit to the MCSA: An exhibit to the MCSA referenced the particular clinical study that the parties agreed to work together on, and set forth that the parties' work on that study was governed by the overall provisions of the MCSA, specifically including the confidentiality provisions thereof.

- Clinical Study Protocol: Once the MCSA was in place and the study site and physicians began working with Alexion to carry out a study, Alexion shared with the site and the physicians the Clinical Study Protocol, which set forth the exact procedure that the participating physician agreed to follow in conducting the study. The Clinical Study Protocol included requirements of confidentiality as well as requirements that the physical study drug be kept in a secure, limited-access location and be carefully tracked via a specific drug accountability logging procedure.

- Consulting Agreement: Often times Alexion also had a previous or co-existing consulting agreement in place with the physicians participating in the clinical study. This agreement set forth the terms that Alexion and the physician agreed to abide by in working with one another to discuss one another's thoughts and ideas as well as study results. The consulting agreement included another requirement that the physician maintain all of Alexion's proprietary information in confidence.

6. As one specific example of how all of these agreements fit together in one particular study, the pilot PNH study of 11 patients that is referenced in D1 to D5 had several layers of confidentiality built into it. (This pilot study is referred to as the "C02-001 Study.") First, there was a Master Clinical Study Agreement ("MCSA") in place between Alexion and each participating institution or study site that required the institution to maintain Alexion's information, which included information about the study drug eculizumab, in strict confidence. As an example, attached as Exhibit C is the MCSA governing C02-001 that was entered into between Alexion and Leeds Teaching Hospitals in the UK, a participant in the C02-001 study. Paragraph 7 of the MCSA states:

   "Institution agrees, and shall cause each Principal Investigator and its employees and agents, not to disclose to any third party and not to use for any purpose other than conducting a Study 1) information that is disclosed by or on behalf of Alexion to Institution or the relevant Principal Investigator and 2) data, inventions, and discoveries which are generated as a result of any Study or that otherwise relate to a Study Drug....Institution agrees to return confidential information of Alexion, and all copies thereof, at the conclusion of each Study or at an earlier date at the request of Alexion..."

   The MCSA also states that the confidentiality provisions "shall survive the termination or expiration of this Agreement."

7. In addition to the express language of the MCSA, Exhibit II to the MCSA (attached as Exhibit D) further describes Study C02-001 that is being conducted pursuant to the MCSA and sets forth additional conditions regarding the Study. Paragraph 8 of this Exhibit specifically references the confidentiality provisions of the MCSA to again highlight these obligations to the signing investigator and his/her institution. Paragraph 8 states: "The Principal Investigator agrees to be bound by the terms of the Master Clinical Study Agreement between Alexion and Institution dated April 1, 2002...Principal Investigator agrees to...the confidentiality provisions of Section 7 of the Master Clinical Study Agreement."

8. Providing a second separate layer of confidentiality requirements, the clinical trial protocol itself, which every investigator participating in the study had to sign and agree to follow, contained strict confidentiality provisions. The protocol governing the C02-001 Study is attached as Exhibit E. Not only is the protocol itself marked "CONFIDENTIAL," but Section 18 of the protocol states:

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

9. Even further, Alexion often had a Confidential Disclosure Agreement ("CDA") in place with the study investigators. For example, Alexion had a CDA in place with Peter Hillmen of Leeds Teaching Hospitals during Study C02-001. This CDA is attached as Exhibit F. Paragraph 2 of the CDA states:

"The Receiving Party agrees not to (1) disclose to any third party any or all of the Information, or permit any third party to have access to such information, or (2) use such Information for any purpose other than to evaluate the Information in connection with discussions between the parties. In addition, the Receiving Party agrees to disclose the Information only to those of its employees who shall reasonably need to know the information in order to evaluate the Information and who are bound to terms of confidentiality that are substantially as protective as the terms hereof."

10. Additionally, Alexion and the institution participating in the clinical study (or the organization providing the institution's financial support) also typically signed a "Form of Indemnity for Clinical Studies" that again acknowledged that the investigators at the institution were required to carry out the study in accordance with the study protocol which, as explained above, included strict confidentiality requirements. Attached as Exhibit G is this Form of Indemnity for Clinical Studies between Alexion and The Leeds Teaching Hospitals NHS Trust regarding the C02-001 Study.

11. Thus, for the C02-001 Study, with respect to Dr. Peter Hillmen and his institution (Leeds Teaching Hospitals), there were at least <u>five</u> times that confidentiality was required, or the investigator and/or study site were reminded of the required confidentiality terms, before the investigator and/or the site were provided with the eculizumab study drug: in the MCSA, Exhibit II to the MCSA, the C02-001 Study protocol, a separate CDA with Dr. Hillmen, and the Form of Indemnity.

<u>Restricted Access to Study Drug</u>

12. Not only were the investigators and institutions that participated in clinical trials repeatedly legally bound to maintain the confidentiality of Alexion's information, but the investigators and institutions also agreed to abide by the strict requirements set forth in the clinical trial protocol regarding the tracking, logging, storage and disposal of study drug material such as eculizumab. For example, as shown in Section 6.6 of the clinical trial protocol for the C02-001 Study (Exhibit E), the receiving investigator or institution was required to maintain an original Receipt of Shipment form upon receipt of study drug material, and then complete a drug accountability ledger that was included in each drug shipment in order "to assist the pharmacist in maintaining current and accurate inventor records covering receipt, dispensing, and the return of study drug supplies." The log required: the identification numbers and initials of subjects to whom the drug was dispensed, the date(s) the drug was dispensed to the subjects, and the date(s) and the number of returned drug vial. Empty vials and vials with residual materials, as well as the inventory records, were required to be kept for later inspection. In addition, all used, partially used and unused vials were required to be documented in terms of how they were disposed of on-site. For example, Exhibit H shows a sample "Drug Disposal Form," which served to document how much drug substance material remained after each administration and/or trial and certified that the institution properly destroyed of any remaining material, e.g., via incineration.

13. In addition, each protocol specified that the study drug be stored in an area with restricted access. For example, Section 6 of the protocol for the C02-001 Study states that, "[a]s with all investigational drugs, Eculizumab must be stored in a secure, limited access storage area." This careful tracking and limited-access storage that was required for all of the eculizumab study drug that Alexion provided for the study ensured that no eculizumab made its way out of the hands of those conducting the study (and who were bound to keep all Alexion information in strict confidence).

14. The C02-001 Study was conducted with 11 PNH patients at two institutions – Leeds Teaching Hospitals and St. George's Medical School, both in the UK. Above I have provided in Exhibits C-G the various documents binding Leeds Teaching Hospitals and its investigators to strict confidentiality and drug accountability and storage provisions. I now attach the corresponding exhibits for the second institution, St. George's Medical School: Exhibit II to the MCSA (attached as Exhibit I, which itself also indicates that an MCSA with similar confidentiality provisions was in place between Alexion and St. George's Medical School) and the Form of Indemnity for Clinical Studies (attached as Exhibit J), in which the St. George's Medical School agreed to abide by the provisions of the C02-001 Study protocol, including the confidentiality and drug accountability and storage provisions I have explained above.

15. I note that all of the authors on references D1, D2, D3 and D5 were affiliated with either Alexion (Leonard Bell, Russell Rother, Scott Rollins, Christopher Mojcik, M. Bombara and B. Petro), Leeds Teaching Hospitals in the UK (Peter Hillmen, Anita Hill, C. Hall, M. Cullen and S.J. Richards), or St. George's Hospital Medical School in the UK (J.

Marsh, D. Elebute and J. Chan). Thus, all authors on these references were bound at least by their own employment agreement confidentiality requirements (at Alexion), or by at least the confidentiality obligations that their respective institutions agreed to abide by in an MCSA. D4 is more of a review article summarizing all of the studies that had been conducted using eculizumab, and the author indicated that the "Source" for the paper was Alexion Pharmaceuticals, Inc.

16. While I have only provided the documents relating to the C02-001 Study, similar documents would have been executed for all of the other eculizumab-related studies conducted by Alexion in the late 1990s and early 2000s based on my experience and knowledge of Alexion's best practices in clinical study initiation and maintenance at that time. I am simply using C02-001 as a representative example in this declaration.

17. Given the multiple layers of confidentiality requirements governing each eculizumab-related clinical trial in the late 1990s and early 2000s, the requirements of all institutions and their principal investigators to account for all study drug material and maintain such material in a secure, limited-access storage area, and the nature of the administration of eculizumab such that patients would never have had direct access to the drug, no member of the public should have been able to obtain Alexion's confidential information and/or study drug material (eculizumab) in the course of any of these clinical studies.

DECLARED at New Haven, CT
United States of America

This 2nd Day of July 2019

Michael Bombara

CURRICULUM VITAE

Employee Name	MICHAEL BOMBARA

EMPLOYMENT HISTORY

Current Employer	Alexion Pharmaceuticals, Inc.
Dates of Employment	May 2017 to Present
Title	Executive Director, Clinical Business Operations Lead
Current Manager	Moke Sharma
Current Employer	Alexion Pharmaceuticals, Inc.
Dates of Employment	March 2016 to May 2017
Title	Clinical Business Operations Lead
Current Manager	Moke Sharma
Current Employer	Alexion Pharmaceuticals, Inc.
Dates of Employment	October 2013 to Present
Title	Clinical Business Operations Lead
Current Manager	Mike Collins
Name of Employer	Alexion Pharmaceuticals, Inc.
Dates of Employment	July 2008 to October 2013
Title	Senior Director, Global Clinical Operations
Manager	Camille Bedrosian and Mike Collins
Name of Employer	Alexion Pharmaceuticals, Inc.
Dates of Employment	January 2007 to July 2008
Title	Director, Clinical Operations
Manager	Stephen Squinto and Camille Bedrosian
Name of Employer	Alexion Pharmaceuticals, Inc.
Dates of Employment	August 2004 to January 2007
Title	Associate Director, Clinical Operations
Manager	Christopher Mojcik
Name of Employer	Alexion Pharmaceuticals, Inc.
Dates of Employment	July 2001 to August 2004
Title	Clinical Project Manager
Manager	Christopher Mojcik
Name of Employer	Alexion Pharmaceuticals, Inc.
Dates of Employment	October 1998 to July 2001
Title	Clinical Project Leader
Manager	Christopher Mojcik

Project Lead for the Following Clinical Studies:

ALXN1210 APAC Regional Lead

ALXN1210 Clinical Operations Lead June 2015 – April 2016

C11-001: An Open Label, Multi-Center Trial of Eculizumab in Patients with Shinga-Toxin producing Escherichia Coli Hemolytic-Uremic Syndrome (STEC-HUS)

E05-001: A Phase 3, Open-label, Extension Study of Eculizumab in Patients With Transfusion Dependent, Hemolytic Paroxysmal Nocturnal Hemoglobinuria (PNH) Who Have Participated in the TRIUMPH (C04-001), SHEPHERD (C04-002), or X03-001 Studies

C04-002: SHEPHERD: Safety in Hemolytic PNH Patients Treated With Eculizumab: A Multicenter Open-label Research Design Study

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CURRICULUM VITAE

Employee Name	MICHAEL BOMBARA

C04-001: TRIUMPH: A Hemoglobin Stabilization and Transfusion Reduction Efficacy and Safety Clinical Investigation, Randomized, Multicenter, Double-Blind, Placebo-Controlled, Using Eculizumab in Paroxysmal Nocturnal Hemoglobinuria Patients

E02-001: An Open-label Extension Study of h5G1.1-mAb (Eculizumab) in Patients With Transfusion Dependent, Hemolytic, Paroxysmal Nocturnal Hemoglobinuria Who Previously Participated in Study C02-001

C02-001: An Open-Label Study of the Effect of Eculizumab (h5G1.1-mAb) on Patients with Transfusion-Dependent, Hemolytic, Paroxysmal Nocturnal Hemoglobinuria

E01-004: Final Report for a Phase 2b Randomized, Double-Blind, Placebo-Controlled Study of the Effect of Eculizumab (h5G1.1-mAb) on Rheumatoid Arthritis (RA) Patients with Active Disease Despite Receiving Methotrexate or Leflunomide

C01-004: Final Report for a Phase 2b Randomized, Double-Blind, Placebo-Controlled Study of the Effect of Eculizumab (h5G1.1-mAb) on Rheumatoid Arthritis (RA) Patients with Active Disease Despite Receiving Methotrexate or Leflunomide

C99-006: A Randomized, Third-party Blind, Placebo-Controlled Pilot Study of the Effect of h5G1 .I-mAb on Dermatomyositis Patients

E99-001: Open-Label Study of the Long-Term Safety of Eculizumab (h5G1.1-mAb) in Rheumatoid Arthritis Patients Receiving Methotrexate who Previously Participated in Study C99-001

C99-001: A Phase 2 Randomized, Double-Blind, Placebo-Controlled Study of the Effect of Eculizumab (h5G1.1-mAb) on Rheumatoid Arthritis Patients Receiving Methotrexate

C97-002: Phase 1 Study of h5G1.1-mAb in Patients With Systemic Lupus Erythematosus

C97-001: Phase 1 Study of h5G1.1-mAb in Patients With Rheumatoid Arthritis

EMPLOYMENT HISTORY (cont.)

Name of Employer	Bayer Corporation, Metabolics & Rhuematic Research
Employer Address (City, State, Country)	West Haven, CT, USA
Dates of Employment	December 1996 to October 1998
Title	Medical Research Associate
Name of Employer	Bayer Corporation, Institute of Bone and Joint Disease & Cancer
Dates of Employment	October 1994 to December 1996
Title	Research Scientist
Name of Employer	Bayer Corporation, Institute of Bone and Joint Disease & Cancer
Dates of Employment	July 1990 to October 1994
Title	Senior Associate Research Scientist
Name of Employer	Bayer Corporation, Institute of Bone and Joint Disease & Cancer
Dates of Employment	December 1988 to July 1990
Title	Associate Research Scientist
Name of Employer	Genentech Incorporated, Immunology
Employer Address (City, State, Country)	South San Francisco, CA, USA
Dates of Employment	September 1986 to December 1988
Title	Research Assistant
Name of Employer	Genentech Incorporated, Bioassay Services
Dates of Employment	August 1984 to September 1986
Title	Laboratory Assistant

EDUCATION

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CURRICULUM VITAE

Employee Name	MICHAEL BOMBARA

Degree, Major	BS, Microbiology
Year Degree Conferred	1984
School	University of Maine
Address of School (City, State, Country)	Orono, Maine, USA

Signature: _____ **Date:** 26 Mar 2019

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Exhibit B

Study Name	Study Number	References	Explicit Confidentiality Provision in Clinical Study Protocol
An Open-Label Study of the Effect of Eculizumab (h5G1.1-mAb) on Patients with Transfusion Dependent, Hemolytic, Paroxysmal Nocturnal Hemoglobinuria Extension Study of Eculizumab in Patients with Transfusion Dependent, Haemolytic, Paroxysmal Nocturnal Haemoglobinuria Who Previously Participated in Study C02-001 An Open-Label Extension Study of Eculizumab in Patients with Transfusion Dependent, Haemolytic, Paroxysmal Nocturnal Hemoglobinuria Who Previously Participated in Study E02-001	C02-001 E02-001 X03-001	D1, D2, D3, D4, D5	Yes
A Randomized, Double-Blind, Placebo-Controlled Pilot Study of the Effect of h5G1.1-mAb on Dermatomyositis Patients	C99-006	D4	Yes
Phase I Study of h5G1.1-mAb in Patients with Rheumatoid Arthritis	C97-001	D4	Yes
A Phase II Randomized, Double-Blind, Placebo-Controlled Study of the Effect of h5G1.1-mAb on Rheumatoid Arthritis Patients Receiving Methotrexate	C99-001	D4	Yes
A Phase IIB Randomized, Double-Blind, Placebo-Controlled Study of the Effect of h5G1.1-mAb on Rheumatoid Arthritis (RA) Patients with Active Disease Despite Receiving Methotrexate or Leflunomide	C01-004	D4	Yes
A Phase II Randomized, Double-Blind, Placebo-Controlled Study of the Effect of h5G1.1-mAb on the Reduction of Proteinuria in Patients with Idiopathic Membranous Glomerulopathy An Open Label Study of the Long-Term Safety of h5G1.1-mAb on the Reduction of Proteinuria in Patients with Idiopathic Membranous Glomerulopathy	C99-004 E99-004	D4	Yes
Phase I Study of h5G1.1-mAb in Patients with Systemic Lupus Erythematosus	C97-002	D4	Yes

Alexion Pharmaceuticals Inc.
352 Knotter Drive ▲ Cheshire, CT ▲ 06410 ▲ Phone 203-272-2596 ▲ Fax 203-271-8196 ▲ www.alxn.com

MASTER CLINICAL STUDY AGREEMENT

THIS AGREEMENT is made as of this 1st day of April, 2002 between The Leeds Teaching Hospitals NHS Trust located at Department of Haematology , Great George Street, Leeds LS1 3EX, UK ("Institution"), and Alexion Pharmaceuticals, Inc., a Delaware corporation, located at 352 Knotter Drive, Cheshire, CT 06410 ("Alexion");

WHEREAS, Alexion may from time to time desire Institution to conduct clinical studies (each, a "Study"), of one or more drug candidates (each, a "Study Drug"), pursuant to mutually agreed protocols which shall be incorporated herewith as Exhibit I (each, a "Protocol"), at Institution's facilities through the principal investigator (each, a "Principal Investigator") referred to on mutually agreed study specific exhibits incorporated herewith as Exhibit II (each, a "Study Specific Exhibit"); and

WHEREAS, by executing any Study Specific Agreement, Institution represents it is equipped to undertake the relevant Study, and Institution shall perform such Study on the terms and conditions as hereinafter set forth;

NOW, THEREFORE, in consideration of the mutual promises expressed herein, each bargained for and given in exchange for the other, the parties agree as follows:

1. **STUDY INITIATION:** Each Protocol will become effective when signed by Alexion and the relevant Principal Investigator. Each Study Specific Exhibit will become effective when signed by Alexion, the Institution and the relevant Principal Investigator.

2. **DATA AND INVENTIONS:** Institution agrees that all data, inventions, and discoveries generated during the course of a Study by Institution, any Principal Investigator or any employee or agent of Institution, or which otherwise relates to a Study Drug shall belong solely and exclusively to Alexion. Institution agrees to execute (and cause each such Principal Investigator, employee or agent to execute) any documents or undertake any further actions if requested by Alexion to evidence transfer of title thereto.

3. **SCOPE OF STUDY:** Institution agrees to exercise, and to cause each Principal Investigator to exercise his or her reasonable and diligent efforts and professional expertise to complete each Study in accordance with the relevant Protocol in a professional, efficient and timely manner. No other investigator may be substituted for the Principal Investigator on any Study, but the Principal Investigator may utilize the services of associates, including co-investigators, employed by the Institution as required; provided, however, that the Institution and Principal Investigator shall remain liable for the acts and omissions of such associates.

4. **TERM:** Subject to earlier termination pursuant to Section 6, the term of this

Agreement shall commence on the date that it is made and continue until the later of (i) December 31, 2005 and (ii) the completion of any Study that remains ongoing as of December 31, 2005.

5. **FINANCIAL CONSIDERATION AND PAYMENT SCHEDULE:** Payment terms and schedules for each Study shall be included in each relevant Study Specific Exhibit.

6. **TERMINATION:** Any Study may be terminated by Alexion at any time in the exercise of its sole discretion upon fifteen (15) days prior written notice to Institution. Any Study may be terminated by Institution upon fifteen (15) days prior written notice to Alexion in the case of significant patient safety concerns. Upon receipt or giving of notice, as the case may be, Institution agrees promptly to terminate conduct of such Study to the extent medically permissible for all patients. In the event of termination of a Study hereunder, other than as a result of a material breach by Institution or the relevant Principal Investigator, the total sums payable by Alexion pursuant to this Agreement in respect of such Study shall be equitably prorated for actual work performed to the date of termination, with any unexpended funds previously paid by Alexion to Institution being refunded to Alexion. Either Institution or Alexion may terminate this Agreement upon written notice to the other at any time in which no Study is ongoing.

7. **CONFIDENTIALITY AND PUBLICATION:** Institution agrees, and shall cause each Principal Investigator and its employees and agents, not to disclose to any third party and not to use for any purpose other than conducting a Study 1) information that is disclosed by or on behalf of Alexion to Institution or the relevant Principal Investigator and 2) data, inventions, and discoveries which are generated as a result of any Study or that otherwise relate to a Study Drug. The preceding obligations shall not apply to information, data, inventions, or discoveries which have entered the public domain through no fault of Institution, or where Alexion agrees in writing to its use or disclosure or which was known by Institution prior to its date of disclosure by Alexion hereunder. Institution agrees to return confidential information of Alexion, and all copies thereof, at the conclusion of each Study or at an earlier date at the request of Alexion, except with respect to raw research Study data where original data will be retained if required by United States Food and Drug Administration regulations.

   Notwithstanding the foregoing, Principal Investigator may publish Study results, subject to the direction of Alexion's multi-center publication committee (if one has been constituted for the relevant Study) and provided a copy of any proposed verbal or written publication is received by Alexion at least 45 days in advance of submission for publication for review and comment on the appropriateness of the data analysis and presentation. If the parties disagree concerning the appropriateness of the data analysis and presentation, Institution and Principal Investigator agree to meet with Alexion representatives at the clinical study site or as otherwise agreed, prior to submission for publication, for the purpose of making good faith efforts to discuss and resolve any issues or disagreement. Alexion may

cause Principal Investigator to delay any such verbal or written publication for 60 days following its receipt if Alexion determines that such period is necessary or desirable to protect its rights to any intellectual property described therein. Furthermore, Alexion may cause Principal Investigator to remove any Alexion confidential information from such proposed verbal or written publication.

The provisions in this Section shall survive the termination or expiration of this Agreement.

8. **REPORTING OF DATA:** Institution agrees to provide Alexion periodically and in a timely manner during the term of this Agreement all the data called for in each Protocol on properly completed case reporting forms. Institution agrees to notify Alexion at the earliest time possible after learning of an adverse experience or drug reaction of any patient in any Study.

9. **COMPLIANCE WITH APPLICABLE LAWS:** Institution agrees to conduct each Study and maintain records and data during and after the term of this Agreement in compliance with all applicable legal and regulatory requirements, including without limitation, any applicable requirements of the United States Food and Drug Administration, including 21CRF Part 312 Subpart D.

Institution shall cause the relevant Principal Investigator to obtain informed consent forms from all subjects participating in each Study. The parties agree that Institution and/or Principal Investigator shall be responsible for the contents of the informed consent forms and shall have the right to modify such informed consent forms in a manner consistent with 21 CFR Part 50 Subpart B, as otherwise required by law and as requested by Institution's IRB. Institution and Principal Investigator agree not to include representations or obligations of Alexion in any informed consent forms.

10. **MONITORING OF STUDY:** During the term of this Agreement, Institution agrees to permit representatives of Alexion to examine at any reasonable time during normal business hours 1) the facilities where any Study is being conducted, 2) raw study data, and 3) any other relevant information (and to make copies) necessary for Alexion to confirm that each Study is being conducted in conformance with the relevant Protocol and in compliance with all laws and regulations, including those of the United States Food and Drug Administration. Original copies of each Study participant's individual medical records shall remain the property of Institution.

Alexion may contract with a third party to coordinate and monitor the conduct of all or part of any Study on behalf of Alexion, and Institution will cooperate with such third party to the same extent as it is obligated to cooperate with Alexion.

11. **PUBLICITY:** Neither party shall use the name of the other party or the name of the other party's affiliated companies, employees, or products for promotional purposes in connection with this Agreement without the prior written consent of the party

whose name is proposed to be used. Notwithstanding the foregoing, Alexion shall be permitted to state orally and in writing the fact that a Study is being, or has been, conducted at Institution under the direction of the relevant Principal Investigator.

12. **INDEPENDENT CONTRACTOR:** Institution and each Principal Investigator is acting in the capacity of an independent contractor hereunder and not as an employee or agent of Alexion.

13. **CONTROLLING LAW:** This Agreement shall be governed by and construed in accordance with English law (other than provisions relating to conflicts of laws).

14. **AGREEMENT MODIFICATIONS:** This Agreement may not be altered, amended, or modified except by a written document signed by all parties.

15. **INCONSISTENCIES:** In the event of any conflict or inconsistency between the terms and conditions of this Agreement and the terms and conditions of any Protocol, Study Specific Exhibit or other documents relating to any Study, the terms and conditions of this Agreement shall govern.

16. **NOTICE:** All notices given hereunder shall be in writing and shall be delivered by hand, facsimile, certified or registered mail, or overnight delivery by a nationally recognized carrier, addressed to the parties as follows:

To: Alexion: Alexion Pharmaceuticals, Inc.
352 Knotter Drive
Cheshire, CT 06410
Attn: Clinical Accounts Associate
Facsimile #: 203-271-8196

Copy To: Alexion Pharmaceuticals, Inc.
352 Knotter Drive
Cheshire, CT 06410
Attn: Vice President and General Counsel
Facsimile #: 203-271-8198

To: Institution: The Leeds Teaching Hospitals NHS Trust
Department of Haematology
Great George Street
Leeds LS1 3EX, UK

Notice shall be deemed given when received and the parties may change the respective addresses where notice is to be given upon prior notice hereunder.

17. **INDEMNITY:** Alexion agrees to indemnify, hold harmless, and defend Institution, Principal Investigator, co-investigator(s), its trustees, regents, directors, officers,

employees, physicians, and all other qualified personnel working under their supervision (collectively, the "Indemnitees") from and against all claims, demands, actions, and proceedings (collectively, "Claims") which may be brought or asserted against Indemnitees to recover damages and losses for or attributable to bodily injury, sickness, disease, or death arising directly and solely out of any Study.

Alexion does not, however, agree to indemnify, hold harmless, or defend Indemnitees from Claims resulting from:

a. failure of any Indemnitee to evaluate or properly interpret available information that is relevant to any Study and for independent decisions made as a result of such failure;

b. failure of any Indemnitee to adhere to all provisions of this Agreement and the Protocol for any Study or to written recommendations and written instructions delivered to such Indemnitee by or on behalf of Alexion concerning the administration and use of any drug substances, including placebo, involved in such Study; or

c. failure of any Indemnitee to render professional service or to conduct any Study in a normal, prudent manner.

A condition of this indemnity obligation is that, whenever an Indemnitee has information from which it may reasonably conclude an incident of bodily injury, sickness, disease, or death is likely to occur or has occurred, such Indemnitee shall immediately give notice to Alexion of all pertinent data surrounding such incident, and in the event a Claim is brought or asserted, such Indemnitee shall assist Alexion and cooperate in the defense thereof, including by gathering information with respect to the time, place, and circumstances and in obtaining the names and addresses of the injured parties and available witnesses. No Indemnitee may, except at its own cost, voluntarily make any payment or incur any expense in connection with any Claim without the prior written consent of Alexion.

The Institution and Principal Investigator shall maintain professional liability insurance as shall be reasonably necessary to insure themselves against any claims for damages whether arising by reason of loss of property, personal injuries or death occasioned in connection with the Study. The ~~Institution~~ and Principal Investigator shall provide a Certificate of Insurance evidencing such coverage to Alexion upon request.

Subject to its internal policies regarding self-insurance, Alexion shall maintain such comprehensive general liability and products liability insurance as shall be reasonably necessary to insure itself against claims for damages arising out of the Study. Alexion shall provide a Certificate of Insurance evidencing such coverage to the Institution upon request.

Institution will ensure that the Study is approved by the Trust Management and that all Managerial and Ethical approval has been obtained, and accordingly that all medical and other staff participating in the Study will be covered by NHS indemnity.

18. **GENERIC DRUG ENFORCEMENT ACT OF 1992:** Institution represents that it and its employees, affiliates, and agents working on each Study have never been 1) debarred or 2) convicted of a crime for which a person can be debarred, under Section 306(a) or 306(b) of the Generic Drug Enforcement Act of 1992 ("Section 306(a) or (b)"). Institution represents that it has never been and, to the best of its knowledge after due inquiry, none of its employees, affiliates, or agents working on each Study has ever been 1) threatened to be debarred or 2) indicted for a crime or otherwise engaged in conduct for which a person can be debarred, under Section 306(a) or (b). Institution agrees that it will promptly notify Alexion in the event of any such debarment, conviction, threat, or indictment. The terms of the preceding sentence shall survive the termination or expiration of this Agreement.

19. **FINANCIAL DISCLOSURE BY INVESTIGATORS**: Institution shall cause each Principal Investigator and each other investigator or sub-investigator, if any, listed in the Form 1572 or identified in the Protocol and directly involved in the treatment or evaluation of research subjects in connection with any Study to complete and deliver to Alexion a financial disclosure questionnaire sufficient to allow Alexion to comply with 21 CFR Part 54. Institution shall cause such persons to respond promptly to any requests by Alexion for additional information or supporting documentation relating to the responses on such financial disclosure questionnaire(s).

IN WITNESS WHEREOF, the parties hereto have caused this Agreement to be executed by their duly authorized representatives as of the date first above written.

ALEXION PHARMACEUTICALS, INC.

By: _____
Name: Thomas I. H. Dubin
Title: Vice President and General Counsel

The Leeds Teaching Hospitals NHS Trust

DR. D. R. NORFOLK
ASSOCIATE R & D DIRECTOR
LEEDS TEACHING HOSPITALS NHS TRUST

By: _____
Name: Dr. D. R. Norfolk.
Title: Associate R&D Director
30/4/02

EXHIBIT I
PROTOCOL
(SUBJECT TO CHANGE WITH EACH STUDY)

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EXHIBIT II
STUDY SPECIFIC EXHIBIT
(SUBJECT TO CHANGE WITH EACH STUDY)

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Alexion Pharmaceuticals Inc.
352 Knotter Drive ▲ Cheshire, CT ▲ 06410 ▲ Phone 203-272-2596 ▲ Fax 203-271-8196 ▲ www.alxn.com

Alexion Pharmaceuticals, Inc.
EXHIBIT II, ATTACHED TO MASTER CLINICAL STUDY AGREEMENT, FOR SPECIFIC STUDY SCOPE, BUDGET & PAYMENT SCHEDULE
with The Leeds Teaching Hospitals NHS Trust

Study Specifications

Upon signature, this Exhibit will be incorporated into and made part of the Master Clinical Study Agreement between Alexion Pharmaceuticals, Inc. ("Alexion") and The Leeds Teaching Hospitals NHS Trust ("Institution") dated, April 1, 2002

1. Product/Compound: h5G1.1-mAb ("Study Drug")

2. Protocol # and Title: C02-001, entitled: "An Open-Label Pilot Study of the Effect of Eculizumab (h5G1.1-mAb) on Patients with Transfusion Dependent, Hemolytic, Paroxymal Nocturnal Hemoglobinuria" ("Study").

3. Principal Investigator: Peter Hillman, MD

4. Contract Research Organization (if applicable, if not put N/A): Kendle

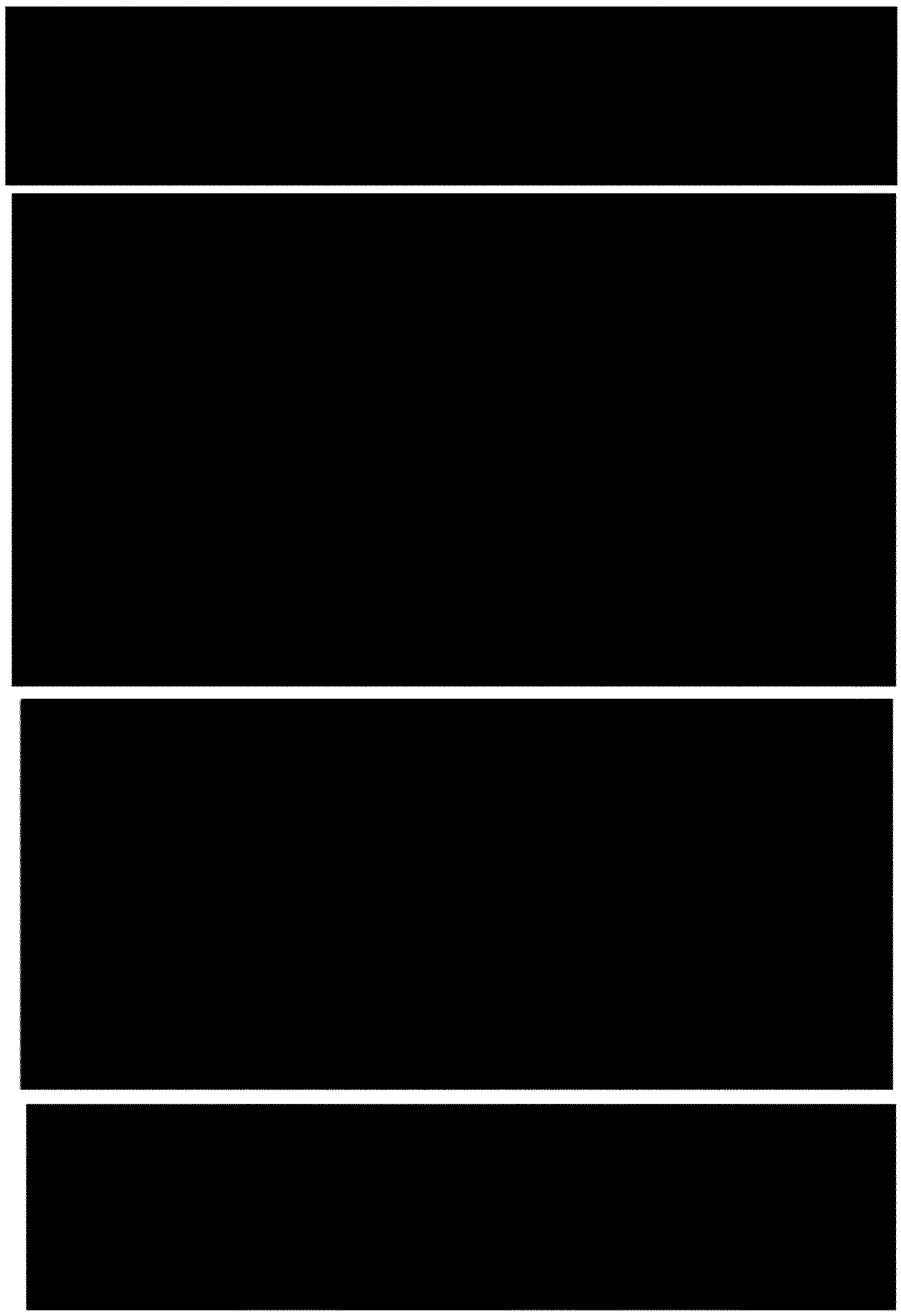
5. Scope:

   The Study will begin following IRB approval and receipt by Alexion of relevant Study documents. The Study will begin around May 1, 2002 and the anticipated completion date is December 31, 2003. Such dates may be modified by written agreement executed by Principal Investigator and authorized representatives of both Institution and Alexion.

6. Subjects:

   The anticipated number of completed and valid subjects in the Study shall be enrolled within the time frame described above, and according to the criteria listed in the Protocol.

[redacted]



7.2 Definitions:

A "Qualified Study Subject" is a research subject who has been randomized and has met the entrance criteria as defined in the Protocol or who is given written permission by Alexion to enter the Study.

A "Protocol Compliant Study Subject" is a Qualified Study Subject who has met the minimum attendance and compliance standards in the Protocol for evaluation of effectiveness of the Study Drug, and has completed the Study.

A "Non-Protocol Compliant Study Subject" is a Qualified Study Subject that violates the Study procedures, such as, insufficient clinic attendance, poor protocol compliance or other protocol violations, as defined in the Protocol, but the subject completes the Study.

An "Early-Terminated Study Subject" is a Qualified Study Subject who has been withdrawn from the Study because of: lack of efficacy, adverse event, lost to follow-up, or voluntary patient withdrawal. Additional follow-up may be required for these patients to meet FDA regulations.

A "Screen Failure Study Subject" is a study subject who meets all entrance criteria and consents to enroll in the study but fails to randomize due to, e.g., unacceptable laboratory values at the screening visit.

A "Non-Qualified Study Subject" is a research subject who has not met the entrance criteria as defined in the Protocol.







8. Provisions of Master Clinical Study Agreement

The Principal Investigator agrees to be bound by the terms of the Master Clinical Study Agreement between Alexion and Institution dated, April 1, 2002. Without limiting the foregoing, the Principal Investigator agrees to the intellectual property provisions of Section 2 of the Master Clinical Study Agreement and the confidentiality provisions of Section 7 of the Master Clinical Study Agreement.

9. Notices

All communications and notices required or permitted under this Agreement shall be deemed sufficiently served if hand delivered or served by certified mail, postage prepaid, addressed as follows:

TO ALEXION: Thomas I. H. Dubin
Vice President and General Counsel
Alexion Pharmaceuticals, Inc.
352 Knotter Drive
Cheshire, CT 06410

TO PRINCIPAL INVESTIGATOR:
Peter Hillmen, MD
Algernon Firth Building
Leeds General Infirmary
Great George Street
Leeds LS1 3EX

TO INSTITUTION: The Leeds Teaching Hospitals NHS Trust
Department of Haematology
Great George Street
Leeds LS1 3EX

ACCEPTED BY:

Alexion Pharmaceuticals, Inc.

By: _____
Thomas I. H. Dubin
Vice President and General Counsel

Date: 5-2-02

The Leeds Teaching Hospitals NHS Trust

By: _____
Dr. D.R. NORFOLK
DR. D. R. NORFOLK
ASSOCIATE R & D DIRECTOR
LEEDS TEACHING HOSPITALS NHS TRUST

Date: 18/06/02

The undersigned, as Principal Investigator, hereby agrees to accept responsibility for performance of the Protocol described herein, acknowledges having reviewed this Agreement and agrees to be bound by the terms and conditions stated herein and in the Master Clinical Study Agreement dated, April 1, 2002, which is incorporated herein by reference.

PRINCIPAL INVESTIGATOR

_____
Peter Hillmen, MD

10/JUNE/02
Date

Attachment A
Study Budget

Working Protocol

CONFIDENTIAL

IND NUMBER:	Pending
STUDY NUMBER:	C02-001
VERSION:	2.1
USAN:	ANTI-C5 ANTIBODY
DOSAGE FORM:	PARENTERAL
STUDY TITLE:	AN OPEN-LABEL STUDY OF THE EFFECT OF ECULIZUMAB (h5G1.1-mAb) ON PATIENTS WITH TRANSFUSION DEPENDENT, HEMOLYTIC, PAROXYSMAL NOCTURNAL HEMOGLOBINURIA
CLINICAL DIRECTOR:	CHRISTOPHER F. MOJCIK, M.D., Ph.D.
ADDRESS:	ALEXION PHARMACEUTICALS, INC. 352 KNOTTER DRIVE CHESHIRE, CT 06410
TELEPHONE NUMBER:	203-272-2596 (OFFICE) 203-784-4333 (EMERGENCIES)
PROJECT SPONSOR:	ALEXION PHARMACEUTICALS, INC.
DATE:	MARCH 15, 2002

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

Principal Investigator — Date

Christopher F. Mojcik, MD, PhD — Date 3/15/02

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FIGURE 1

STUDY FLOW CHART

Visit	1	2	3	4	5	6	7	8	9	10	11	12	PT
Day (Month)	-14 to -21	0	7	14	21	28 (1)	35	42	56 (2)	70	84 (3)	112 (4)	
Assessment	SCR	Baseline											
Dosing (mg)		600	600	600	600	900		900	900	900	(900)*	(900)*	
Informed consent	x										(x)*		
Medical history**	x												
Weight	x										(x)*		
Vital signs	x	x	x	x	x	x	x	x	x	x	x	x	x
Physical examination	x										x		x
12-lead ECG	x										x		x
Hematology	x	x	x	x	x	x	x	x	x	x	x	(x)*	x
Blood chemistry	x	x	x	x	x	x	x	x	x	x	x	(x)*	x
Ferritin, Vitamin B12, Folate	x										x		x
Coagulation Profile		x	x	x	x	x			x		x		x
Urinalysis	x	x				x			x		x	(x)*	x
Reticulocyte count		x	x	x	x	x	x	x	x	x	x		x
Haptoglobin		x		x		x		x	x	x	x		x
Plasma (free) hemoglobin		x									x		x
Ham Test		x									x		x
Flow cytometry	x	x	x	x	x	x	x	x	x	x	x		x
EORTC QLQ-C30		x				x			x		x		x
Self assessment urine diary		x	x	x	x	x	x	x	x	x	x		x
Neisserial throat culture	x	x				x			x		x		x
PK/PD/SC5b-9		t/p			t/p	t/p	i	t		t/p	t		t
Anti-h5G1.1-mAb (HAHA)		x				x			x		x		x
Concomitant Medications	x	x	x	x	x	x	x	x	x	x	x	x	x
Adverse events		x	x	x	x	x	x	x	x	x	x	x	x
Hepatitis Screen	x												
Pregnancy (β-HCG)	x										x		x
Neisserial vaccine	x												
Exchange transfusion											x***		x^

Abbreviations: PT = Premature Termination; SCR = Screening; PK = Pharmacokinetics; PD = Pharmacodynamics; t = trough sample; p = peak (1 hour after dosing) sample; i = interval sample. * = Only if participating in extension study. * = Includes laboratory values from the 3 months prior to screening. ***Only if not participating in extension study and PNH RBC's >50% of total RBC's by flow cytometry. ^ Only if PNH RBC's >50% of total RBC's by flow cytometry.

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GLOSSARY OF ABBREVIATIONS
(in alphabetical order)

BUN	=	blood urea nitrogen
CRF	=	case report form
ECG	=	electrocardiogram
GCP	=	good clinical practice
Hct	=	hematocrit
Hgb	=	hemoglobin
IRB	=	institutional review board
LDH	=	lactic dehydrogenase
mmHg	=	millimeters of mercury
OTC	=	over-the-counter
Plt	=	platelet
PMN	=	polymorphonuclear leucocyte (neutrophil)
PNH	=	paroxysmal nocturnal hemoglobinuria
PRBC	=	packed red blood cells
RBC	=	red blood cell
sec	=	seconds
SGOT	=	serum glutamic-oxaloacetic transaminase (AST; aspartate aminotransferase)
SGPT	=	serum glutamic-pyruvic transaminase (ALT; alanine aminotransferase)
ULN	=	upper limit of normal

NOTE ON USAGE OF TERMS:

- The term "Investigator" refers to the principal investigator or responsible physician.
- The term "study staff" refers to the Investigator or associates of the Investigator.
- The term "Pharmacist" refers to the staff member responsible for preparing study medication for dispensing.

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STUDY SYNOPSIS

COMPOUND	h5G1.1-mAb
STUDY TITLE	An Open-Label Study of The Effect of Eculizumab (h5G1.1-mAb) on Patients with Transfusion Dependent, Hemolytic, Paroxysmal Nocturnal Hemoglobinuria.
DEV'T PHASE	Pilot
KEY TARGET DATES	Study Start: May 1, 2002 Last Subject Enrolled: July 31, 2002 Last Subject Completed: November 30, 2002
INDICATION	Subjects to be recruited for this study will be patients with transfusion dependent, hemolytic, PNH.
OBJECTIVES	The primary objective is to determine the safety and tolerability of h5G1.1-mAb and to evaluate the pharmacokinetics and pharmacodynamics of this antibody in the target population.
DESIGN	Open-label.
SUBJECTS	Subjects to be recruited for this study will be patients with transfusion dependent PNH.
SAMPLE SIZE	10 subjects
TREATMENTS	Eculizumab (h5G1.1-mAb) 600 mg via IV bolus over 30 minutes every week for 4 doses, then 900 mg via IV bolus over 30 minutes every other week for 4 doses.
CLINICAL EFFICACY AND SAFETY VARIABLES	Efficacy Variables: Primary-none designated, as this is a pilot study. Secondary-number of units PRBCs transfused; time to first transfusion of PRBCs; change from baseline hemoglobin and hematocrit; change from baseline indicators of hemolysis (reticulocyte count, haptoglobin, LDH and bilirubin); number of paroxysms and time to first paroxysm; reticulocyte count; haptoglobin; change from baseline EORTC QLQ-C30; pharmaco-dynamics. Exploratory-platelet and neutrophil count; flow cytometry; Ham test; plasma (free) hemoglobin Safety Variables: Adverse events, laboratory data, ECGs.
PROCEDURE	Enrollment occurs following satisfaction of entry criteria. Subjects receive study medication as a constant infusion over 30 minutes: 600 mg weekly for 4 weeks and then 900 mg every other week for 6 weeks, for a total of 8 treatments. Study procedures will be followed according to the flow sheet.

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NUMBER OF CENTERS	There will be up to 2 study centers in the United Kingdom.
DURATION OF STUDY PER SUBJECT	16 weeks.
STATISTICAL METHODS	**Efficacy Analysis** Mean change from baseline to post-baseline assessments for the secondary and exploratory endpoints will be summarized by the mean, median, standard deviation and range at each post-baseline assessment for each treatment group and overall. **Safety Analysis** The safety analysis will compare treatment groups with regard to adverse events, laboratory abnormalities and vital signs. Summary statistics will describe each category for laboratory abnormality and vital signs. Adverse events will be tabulated. Analysis of variance will test the difference between treatment groups and either CMH or Fisher's exact test will be employed for categorical data.
SPONSOR	ALEXION Pharmaceuticals, Inc. 352 Knotter Drive Cheshire, CT 06410
CLINICAL RESEARCH ORGANIZATION (CRO)	None.

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1. INTRODUCTION

PNH was first described in 1882 [1], and is an acquired clonal disorder of the hemopoietic stem cell. It is well known for its triad of clinical manifestations: intravascular hemolysis, thrombosis and reduced hematopoiesis [1, 2]. It is an uncommon condition with an incidence of somewhere between 1:100,000 and 1:1,000,000. The disease can present at almost any age (median of approximately 35 years) and with a roughly equal sex distribution [2-5].

The three cardinal features of PNH are hemolysis, cytopenias and thrombosis. There is a continuum from mainly hemolytic in type to predominantly aplastic with a subclinical PNH clone. The clinical characteristics, as well as clone size, may change throughout the course of the disease.

In a third of cases, hemolysis is the predominant symptom. The vast majority of hemolytic PNH patients (>80%) have prominent hemoglobinuria at some point in the disease [2], and a quarter of patients present with dark urine in the early morning. This is usually episodic and precipitated by infection, drugs or trauma (presumably related to complement activation). Hemoglobinuria very occasionally progresses to acute renal failure, which is usually reversible. There may also be esophageal spasm and erectile dysfunction [1], both symptoms of smooth muscle dysfunction, possibly caused by the adsorption of nitric oxide by free plasma hemoglobin.

Cytopenias may affect all lineages of hematopoiesis. Anemia may be associated with iron or folate deficiency as well as evidence of hemolysis. On bone marrow examination, erythroid hyperplasia is usually accompanied by hypoplasias and a variable degree of dyserythropoiesis. Further, there is an association of PNH with aplastic anemia and about one-third of patients have aplastic anemia diagnosed before the onset of PNH [2]. Thrombocytopenia is present in 80% of patients [2]. PNH granulocytes have normal lifespans [1], and unless PNH patients are significantly neutropenic, infections do not occur at an increased rate. This suggests that the membrane abnormalities do not significantly affect neutrophil function. Involvement of lymphocytes is usually minimal and not associated with immunodeficiency.

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Venous thrombosis in PNH occurs in a large minority (39%) of patients, and may be repetitive [2]. It tends to occur in unusual anatomic locations, such as hepatic veins [1]. Further, it often occurs without warning, and is a common cause of mortality for these patients [2, 5].

Flow cytometry is increasingly used for diagnosis and monitoring. Red cells and granulocytes display complete (type III) or partial (type II) deficiency of GPI linked proteins [6]. Type III cells are most sensitive to the effects of complement. (See below for a discussion of GPI-linked proteins and complement.) Patients often show the presence of Type II and Type III cells simultaneously [5]. The proportion of clonal subtypes (e.g. Type II or III cells) tends to predict the clinical phenotype [5]. On the whole, hemolytic patients have large clones (>50% PNH neutrophils) and hypoplastic patients smaller ones (<20% PNH neutrophils and <10% PNH erythrocytes)[7]. It seems that thrombosis, in particular, is related to larger clone size. A recent UK study found that the 10 year cumulative incidence rate of thrombosis was 39% in patients with granulocyte clone sizes of ≥50% but only 7% in those with clone sizes of <50% [8].

Although approximately 15% of PNH will spontaneously remit [2], the prognosis of PNH is generally quite poor. In the series by Dacie and Lewis [9], the median survival after diagnosis was found to be 8 years and in a recent review of the series this was increased to 10 years [2]. In the large retrospective multicenter study in France with 220 PNH patients between 1950 and 1995, the Kaplan Meier survival estimate at 10 years was 65% and at 15 years 48% [4]. 30% of these patients had a previous history of aplastic anemia. PNH is directly implicated in the cause of death in approximately a half of patients. The majority of deaths are due to venous thrombosis, with progressive cytopenias the second most common cause [1, 5].

Therapy for PNH is primarily symptomatic. Anemia prophylaxis with iron and folic acid can be effective, and transfusion should be avoided if possible. A potential problem with iron supplementation is that the resultant reticulocytosis may precipitate hemoglobinuria. Steroids are thought by some to reduce the frequency of attacks but there appears no reason that they should affect either the activation of complement or the proportion of PNH red cells. In one small

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series of five refractory and transfusion dependent patients, four benefited from Danazol and became transfusion independent [10]. Oral anticoagulation is used for prophylaxis of thrombosis, and thrombolytic therapy for acute thrombotic events. Immunosuppression with either anti-thymocyte globulin or Cyclosporine is effective in the 10% of patients with progressive pancytopenia [1, 11]. The only curative treatment is bone marrow transplantation, but due to its substantial morbidity and mortality, this procedure is available to only a small proportion of patients [1, 2].

The biochemical defect in PNH occurs in the synthesis of the glycosyl-phosphatidylinositol (GPI) anchor. This glycolipid structure is the means by which many proteins are attached to the plasma membrane. The *pig-a* (phosphatidyl-inositol glycan complementation class A) gene comprises 6 exons and spans 17 kilobases on the short arm of the X chromosome, at Xp22.1 [1]. PIG-A forms a complex with PIG-H, PIG-C and GPI1 [12] to mediate the first step in the biosynthesis of the GPI anchor. All reported patients with PNH have a haematopoietic GPI-deficient clone due to a somatic mutation of *pig-a* [5]. In fact, many patients have more than one independent GPI deficient clone with up to four being described in a single patient. Constitutional *pig-a* defects have not been described. A single mutation event is sufficient to cause disease because the *pig-a* gene is X-linked.

Unanchored membrane proteins are degraded in the endoplasmic reticulum or expelled from the cell. All GPI linked proteins have been found to be missing from PNH haemopoietic cells. GPI linked proteins have extremely diverse functional roles, including coreceptors, signal transduction, membrane transport, complement receptors, and complement regulation [1]. The two missing proteins thought to cause the clinical manifestations of the disease are DAF (Decay Accelerating Factor; CD55) and MIRL (membrane inhibitor of reactive lysis; CD59). DAF is an inhibitor of C3 and C5 convertases, accounting for the increased deposition of C3b on PNH cells after complement activation . However, patients with the Inab phenotype, in which CD55 is congenitally absent, have only slightly increased sensitivity to complement. It is therefore unlikely that absence of DAF is solely responsible for the intravascular hemolysis of PNH. MIRL is an inhibitor of membrane attack complexes. It binds to the C8 component of complement, preventing the binding and subsequent

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polymerization of C9. In the absence of MIRL, polymeric C9 is able to disrupt the cell membrane and this accounts for the intravascular hemolysis and possibly the thrombosis which occur in PNH. The major role of MIRL is supported by the fact that congenital absence of CD59 is associated with clinical hemolysis, hemoglobinuria and venous thrombosis.

There is an illustrative case report of a woman with C9 (complement component 9) deficiency and PNH. However, her clinical course was clearly distinctive for PNH. Her RBC's were approximately 80% Type III and 95% DAF negative. Despite this, she suffered only mild hemolysis until she received a postoperative blood transfusion (which reversed her C9 deficiency). She then had episodes of gross hemoglobinuria and jaundice, indicative of spontaneous, massive hemolysis [13].

The proinflammatory activities of the activated terminal complement components C5a and C5b-9 have been implicated in a wide range of inflammatory disease states [14]. The complement system is comprised of over thirty serum and cell surface proteins that subserve important roles in immune recognition and effector function [15]. Upstream activation of complement through either the classical or alternative pathways leads to the cleavage of C3 into C3a, a weak anaphylatoxin, and C3b, which is critical for opsonization of pathogenic microorganisms and for the clearance of immune complexes. C3b also participates in the activation of C5, which results in the production of C5a and C5b. C5a is a potent anaphylatoxin, chemotactic factor, and cell activating molecule that mediate multiple proinflammatory activities [16]. C5b recruits the terminal complement components C6-9 to form the terminal complement complex, C5b-9. This complex is cytolytic when present in large quantities, or proinflammatory when present in sublytic quantities. It is capable of disrupting normal homeostasis following deposition on platelets and endothelial cells [14, 17].

Eculizumab (h5G1.1-mAb) is a humanized monoclonal antibody to human C5. It binds to C5 and prevents its cleavage into C5a and C5b. It has been used in single dose Phase I studies in rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) patients. In a dose escalation study of 42 RA patients, h5G1.1-mAb was safe and well tolerated up to 8 mg/kg. There was one serious adverse event (SAE) in the lowest dose cohort, in which a single patient had 2

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flares of his RA, which required hospitalization for treatment with high-dose steroids and plasmapheresis. This SAE was not considered related to study medication by the principal investigator (PI). Otherwise, there were no dose-dependent trends in adverse events noted across dose groups, and there were no clinically significant laboratory abnormalities noted. Serum hemolytic activity (a surrogate of complement activity) was completely blocked for an average of 7 days at the 8 mg/kg dose level (n=6) and did not return to baseline levels until day 14. (Data available on file at Alexion Pharmaceuticals, Inc.)

In 24 SLE patients, a similar dose escalation study was performed. Again, h5G1.1-mAb was safe and well tolerated up to 8 mg/kg. There were three SAEs in this study: 1) a patient in the placebo group suffered a fractured ankle; 2) a patient in the 4 mg/kg dose group had a near-syncopal episode; and 3) a patient in the 8 mg/kg dose group had an exacerbation of asthma. None of these SAEs were considered related to study medication by the PI's involved. Otherwise, there were no dose-dependent trends in adverse events noted across dose groups, and there were no clinically significant laboratory abnormalities noted. Serum hemolytic activity blockade was similar to that seen in the RA study. (Data available on file at Alexion Pharmaceuticals, Inc.)

A Phase IIa, multi-center, double blind, placebo-controlled trial was then performed, testing three dosages of h5G1.1-mAb in patients with rheumatoid arthritis also taking methotrexate. Treatment arms included: 1) placebo (PLC); 2) h5G1.1-mAb 8mg/kg q 2 weeks (Q2wk); 3) h5G1.1-mAb 8mg/kg q week X5, then q month (Induction/Q4wks); 4) h5G1.1-mAb 8mg/kg q week X5, then q 2 weeks (Induction/Q2wks). Thus, the maximum cumulative dose given was 72 mg/kg. There were 209 patients treated for 3 months, then followed for 3 additional months off study medication. The agent showed an inverted U-shaped dose-response curve, with the Induction/Q4 wks regimen showing moderate efficacy (44% ACR 20 vs. 18% placebo rate) at 3 months. In a subpopulation of patients with elevated baseline serum C5b-9 levels, a larger percentage of patients in the active treatment groups responded, and the agent displayed a more conventional dose-response curve. Following the withdrawal of this agent, patients tended to worsen in the majority of clinical measures. There were no safety concerns noted with any of the regimens used. (Data available on file at Alexion Pharmaceuticals, Inc.) Further, this trial was overseen by a data safety

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and monitoring board (DSMB), which periodically reviewed safety data. There were no concerns raised by this panel regarding the trial.

This agent is also being used in a Phase II study of idiopathic membranous nephropathy patients. In this study, patients are treated with Eculizumab for 4 months at dosages of 8 mg/kg every 2 weeks or every 4 weeks, or placebo. The maximum cumulative dose given was also 72 mg/kg. This trial is still blinded, so results are not currently available; however, there appear to be no safety issues to date. As with the RA trial noted above, the membranous trial is monitored by a DSMB. They have raised no safety concerns thus far.

For both the Phase IIa RA study, and the Phase II membranous study, year long extension studies have been offered. One hundred and thirty-two RA patients entered the extension (receiving 8 mg/kg every 2 weeks); to date, 70 patients have completed a year of therapy. Thus, there are over 107 patient-years of exposure in this population. In the membranous group, 60 patients have enrolled; thus far 21 have completed a year. There are over 40 patient-years of exposure in this population.

Across all trials thus far, there has been one incident of meningococcal meningitis. This occurred in a membranous nephropathy patient who had completed the blinded Phase II study and had been on open label (extension study) therapy for 3 months. She recovered with antibiotic therapy and was left with necrosis of several digit tips as sequelae (see section 7, below).

A related compound is h5G1.1-scFv, which has been used in a 24 hour regimen in over 3000 patients in cardiovascular intervention trials. This agent was shown to potently inhibit the generation of activated terminal complement byproducts such as C5b-9, but did not diminish the generation of C3b [18].

If additional information is needed, please refer to the Investigator's Brochure.

2. STUDY OBJECTIVES

The objectives of this study are:

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1. To determine the clinical and laboratory safety and tolerability of Eculizumab administered intravenously (IV) in patients with PNH;
2. To obtain information on the pharmacokinetics and pharmacodynamics of this antibody in the target population.

3. OVERALL DESIGN AND PLAN OF STUDY

This is an Open-Label Pilot study of Eculizumab administered to patients with transfusion dependent, hemolytic, PNH.

There will be 10 patients treated. Following a screening period of between 14 and 21 days, a 10 week, open-label treatment period will commence. Patients will receive Eculizumab (h5G1.1-mAb, 600 mg) by bolus infusion every week for 4 weeks, then Eculizumab (900 mg) by bolus infusion every other week for 6 weeks. All patients will receive 8 infusions of Eculizumab.

Patient safety will be assessed via the collection of adverse events and the evaluation of pre- and post-treatment laboratory data. In addition, various efficacy parameters, e.g., transfusion requirements, blood indices, and EORTC QLQ-C30, will be evaluated.

4. ETHICS AND GOOD CLINICAL PRACTICE

- This study will be conducted in accordance with Alexion standard operating procedures, which are designed to ensure adherence to Good Clinical Practice (GCP) as required by the following:
- Directive 91/507/EEC: The Rules Governing Medicinal Products in the European Community;
- Code of Federal Regulations dealing with clinical studies (21 CFR including parts 50 and 56 concerning informed consent and IRB regulations); and
- Declaration of Helsinki, concerning medical research in humans ("Recommendations Guiding Physicians in Biomedical Research Involving Human Subjects," Helsinki 1964, amended Tokyo 1975, Venice 1983, Hong Kong 1989, Somerset West 1996, and Edinburgh, 2000).

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- Food and Drug Regulations dealing with clinical studies (Part C) and associated Guidance for Clinical Trial Sponsors (draft 2001/08/01)
- Note for Guidance on Good Clinical Practice in valid version approved by CPMP in July 1996.
- International Conference on Harmonization; Good Clinical Practice: Consolidated Guideline; Notice of Availability, in Federal Register, May 9, 1997; adopted by Therapeutic Products Directorate of Health Canada, 1997.
- Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans, Medical Research Council of Canada, Social Sciences and Humanities Research Council of Canada, August 1998

5. STUDY POPULATION

5.1 Primary Diagnosis

The study population will comprise individuals who have transfusion dependent, hemolytic, PNH.

Patients will be treated in accordance with the standard medical practice of the physician charged with their care, with few exceptions: please see Section 6.7, Concomitant Treatment, below.

5.2 Enrollment Criteria

- Age $\geq$ 18 years.
- Transfusion dependent (i.e. at least 4 transfusions in the last 12 months) hemolytic PNH.
- Presence of a GPI deficient clone.
- Disease duration $\geq$ 6 months.
- No new immunosuppressive agents x 84 days prior to first dose of study medication.
- No history of bone marrow transplantation.
- Patient must be willing and able to give written Informed Consent.

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- Patients must be post-menopausal, surgically sterile or practicing a medically approved method of contraception.
- A negative throat culture for N. meningitidis or N. gonorrhoeae. If the screening throat culture comes back positive for these species, the patient must be treated with appropriate antibiotics, and may be re-tested. If the repeat throat culture is negative for these organisms, the patient is then eligible. Study centers must wait for the final throat culture report from the central lab before making the decision as to a patient's eligibility for the study. Patients that test positive for non-pathogenic species of Neisseria may be enrolled as long as there is no history of meningococcal disease or symptoms of active infection.

5.3 Exclusion Criteria

- Liver disease, defined as AST/ALT and bilirubin $\geq$ 1.8 times the upper limit of normal (ULN), not related to PNH, at screening.
- History of alcohol or drug abuse within two years of screening.
- A history of positive Hep B or Hep C serology.
- Renal insufficiency, defined as serum creatinine $\geq$ 2 X ULN.
- History of malignancy, excepting non-melanoma skin cancer and remote ($\geq$ 2 years) malignancies in complete remission.
- Presence or suspicion of active bacterial infection in the opinion of the investigator, or recurrent bacterial infections.
- Known or suspected hereditary complement deficiency.
- Participation in any other investigational drug trial or exposure to other investigational agent, device, or procedure within 30 days prior to screening.
- Pregnant or breast-feeding patients and women intending to conceive during the course of the study, including the follow-up period.
- History of meningococcal disease.
- Poorly controlled diabetes, defined as blood glucose greater than 300 mg/dl, or recent (within 3 months) HbA1c > 10.5%.
- History of allergic reaction to murine proteins.
- Prior exposure to h5G1.1-mAb.

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- Any clinically significant medical condition that, in the opinion of the investigator, is likely to interfere with the patient's participation in the study or the evaluation of the study medication's safety profile.
- Patients who are not vaccinated against N. meningitidis

5.4 Number of Patients

It is estimated that 10 patients will be treated. There will be up to 2 study centers.

5.5 Method of Patient Assignment and Blinding Procedure

None, as this is an open-label study. All patients will receive Eculizumab.

6. DRUGS, DOSAGE AND DURATION OF TREATMENT

6.1 Test Material and Control Agents

Eculizumab (h5G1.1-mAb) 10 mg/ml

6.2 Supply, Packaging, Labeling and Storage

The active study medication is manufactured by Alexion Pharmaceuticals, Inc., and is shipped to Clinical Trial Services, who will supply it to the sites. Each site will supply the appropriate diluent for mixing the study medication.

Eculizumab will be packaged as a solution for injection in 30 ml vials with a concentration of 10 mg/ml. It will be packaged in bulk containers and shipped to the site prior to study start. This bulk shipment will include specific directions as to the dilution at which the infusion will be administered.

Each vial of Eculizumab will have a two-part label describing the vial contents and a place for the pharmacist to record patient number, initials, and treatment number. The tear-off portion of the label is to be affixed to the label sheet provided.

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The site should notify Clinical Trial Services immediately if vials are damaged and cannot be used: Clinical Trial Services will ship additional drug at once. As with all investigational drugs, Eculizumab must be stored in a secure, limited access storage area. When study medication arrives, it should be promptly removed from the shipping cooler and stored in the refrigerator at 2° – 8°C.

6.3 Dosage and Administration

Preliminary pharmacokinetic analyses have been performed using data obtained from Phase II trials in rheumatoid arthritis and psoriasis completed by Alexion wherein patients were given 8.0mg/kg infusions of eculizumab. These analyses compared the actual attained steady-state serum concentrations at a trough and a mid interval between troughs with theoretical concentrations attained with a 600 mg fixed dose calculated from the same individuals. These data demonstrated that the 600mg fixed dose had favorable characteristics compared to dosing on a mg/kg basis. The geometric and arithmetic means for the trough concentrations were very similar (i.e. within 5 μg/ml of each other).

These preliminary analyses support the use of a fixed dose in this setting. There is little potential to overdose with either dosing system because there is no dose-related toxicity associated with this agent noted after treating more than 300 patients. Nevertheless, the fixed dose may produce lower peak values. A more likely problem therefore is the potential to underdose. However the use of a fixed dose of 600 mg appears to be at least at good as the weight based dosing system in obtaining trough concentrations in excess of 30 ug/ml.

Analysis of the pharmacodynamic properties of eculizumab in the Phase II study in Rheumatoid Arthritis patients revealed that serum complement activity was not completely blocked for the entire 12 week treatment period. In this study, the patients in the highest dose group received 8.0mg/kg for 5 weeks followed by biweekly infusions of 8.0mg/kg for 2 months. Serum complement activity was essentially completely blocked in all patients during the weekly dosing period but

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several patients exhibited a modest return of complement levels during the biweekly dosing schedule.

Because the primary lesion in PNH patients results from the lysis of susceptible erythrocytes by serum complement, we propose a modified dosing schedule for this study that should provide more complete blockade of serum complement during the proposed clinical study period. This modified regimen will employ the fixed weekly 600mg dosing schedule (as described above) for 4 weeks followed by a fixed 900mg biweekly dose for 2 months. This modified dosing regimen should increase eculizumab serum levels such that the PNH patient's serum complement levels are more completely blocked during the clinical study period.

Patients will receive 600 mg of Eculizumab every week X 4 doses, then 900 mg of Eculizumab every other week X 4 doses. See the Study Flow Chart on page 4.

All patients will receive 8 infusions of Eculizumab.

The initial dose of study medication will be 600 mg of Eculizumab and will be contained in three (3) 30 ml vials. (Each 30 ml vial contains only 27.5 ml of Eculizumab.) The maintenance dose of study medication will be 900 mg of Eculizumab and will be contained in three (4) 30 ml vials. Patients will be infused with study medication at a concentration of 5 mg/ml, in a volume of 120 mls (600 mg) or 180 mls (900 mg). A one-to-one dilution of the vialed material will be made (one part study medication, one part diluent): e.g. 60 mls study medication to 60 mls diluent. Study medication can be mixed with normal saline, ½ normal saline, or D5W.

The study medication is stable for at least 24 hours once diluted. The medication is stable in the three diluents mentioned above whether it is prepared in standard IV bags or glass evacuated bottles. If the study medication is made up more than several hours in advance of a patient visit, then the diluted material should be stored at 4°C and warmed to room temperature prior to administering the infusion. The solution should be allowed to come to room temperature by taking it

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out of the refrigerator. The material must not be heated in a microwave, nor with any heat source other than ambient air temperature.

The study medication and diluent will be injected into a bag or bottle suitable for infusion. The diluted study medication will then be administered intravenously over 30 minutes. At the site's discretion, the diluted study medication may be placed in a syringe-type pump or infused with an infusion pump. The patients will be monitored for one hour after the end of each infusion.

If an adverse event occurs during the administration of the study medication, the infusion may be slowed or stopped at the discretion of the investigator, dependent upon the nature and severity of the event. If the infusion is slowed, the total infusion time may not exceed two (2) hours.

It is not necessary to protect the infusion bags from light while they are being administered to the patient.

6.4 Duration of Treatment

The duration of treatment with study medication will be 10 weeks.

6.5 Premature Termination of Treatment/Patients From the Trial

If a patient discontinues participation in the trial and has not received any study medication, the patient will be replaced. No further protocol-related procedures need to be performed.

If the patient discontinues participation in the trial after being dosed, regardless if they received any study medication, they will be eligible for the intention-to-treat analysis, and the analysis of non-treatment emergent serious adverse events. Every effort should be made to perform all of the procedures in Section 10.1, under Premature Termination Visit.

Some reasons for the premature discontinuation of a patient from the trial are:

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- Patient wishes to withdraw;
- Patient is unable or unwilling to complete follow-up;
- Patient experiences a serious adverse event.
- Lack of Efficacy: defined as a patient who requires transfusions on two (2) separate occasions after Week 3.

6.6 Drug Accountability

When a drug shipment is received from Clinical Trial Services the investigator or his or her designee will sign the Receipt of Shipment form provided with the shipment and maintain the original copy for collection by the Study Monitor/Research Associate. The duplicate copy should be kept with the pharmacy's dispensing logs.

Each shipment of investigational drug supplies for a study will contain a drug accountability ledger to assist the pharmacist in maintaining current and accurate inventory records covering receipt, dispensing, and the return of study drug supplies. During the course of the study, the following information must be noted in the drug dispensation log: the identification numbers and initials of subjects to whom drug is dispensed, the date(s) that drug was dispensed to subjects, and the date(s) and the number of returned drug vials. Empty vials and vials with residual materials should be kept for inspection by the Study Monitor/Research Associate. The inventory records also must be readily available for inspection by the Study Monitor/Research Associate, and may be subject to FDA or the local regulatory agency inspection at any time.

All used and partially used vials of study medication will be retained until the Study Monitor/Research Associate has reviewed all study drug dispensation logs. The Study Monitor/Research Associate will assure proper documentation exists for disposition of used (empty), partially used and unused vials on-site. The Study Monitor/Research Associate will collect the Drug Dispensing Logs/Inventory Sheets, and copies of these documents will be retained by the investigator for his or her files.

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6.7 Concomitant Therapy

Patients will be treated in accordance with the standard medical practice of the physician in charge of their care, with the following exceptions:

1. Immunosuppressants are allowed, but must be kept stable during the study (Visits 1-12).
2. Iron (Fe) supplements are allowed, but must be kept stable during the study (Visits 1-12).
3. Warfarin is allowed, but may not be started during the study (Visits 1-12).
4. Whole blood or fresh frozen plasma should not be given during the study (Visits 1-12)

All concomitant medications taken by the patient within the thirty days prior to screening will be recorded in the appropriate section of the case report form.

It is recommended that patients are transfused only if their hemoglobin falls to below 10 g/dl and they have symptoms attributable to anemia. Blood for transfusion should not contain complement and hence packed cells in Optimal Additive Solution (OAS) is recommended. Whole blood should not be used.

7. WARNINGS/PRECAUTIONS

To date there have been two cases of urticaria (immediate hypersensitivity reactions) reported during administration of Eculizumab. Both patients experienced a reaction upon receiving their first infusion and both recovered fully after treatment with an antihistamine. Neither patient had hemodynamic instability or breathing difficulties, although one patient had a tingly sensation in the tongue. The patients were discontinued from their respective studies. An acute allergic reaction should be treated according to standard medical practice (e.g., with epinephrine or antihistamine).

Inhibition of the late elements of the complement cascade system may predispose patients to infections with N. meningitidis. There has been one reported case of meningitis with this organism that occurred in a patient with

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membranous nephropathy patient who was enrolled in an open-label extension study. This patient had several risk factors for infection in addition to having been treated with Eculizumab. Following antibiotic treatment, the patient recovered with some sequelae (loss of digits). Therefore, to decrease the risk of such possible infection, all patients in this study must be vaccinated against N. meningitidis. These patients must be vaccinated at least two weeks before his or her first dose of study medication. Additionally, all patients will have frequent throat cultures to detect colonization with N. menigitidis.

In addition to the risks with N. meningitidis discussed above, patients may be at an increased risk for other types of infections. Patients must not be dosed if they have a temperature of 38°C (100.4°F) or greater.

As this is a clinical trial, careful observation of the patient is necessary. Any adverse event experienced during the study period must be described in detail, fully evaluated by the investigator, and reported on the appropriate CRF pages regardless of the investigator's perception of the event's relationship to the study material.

8. PRETREATMENT PROCEDURES

8.1 Informed Consent

Prior to the initiation of any invasive testing or protocol mandated procedure, the study will be explained to the patient. If the patient agrees to all of the requirements of the study, and to the Informed Consent, the patient will sign the Informed Consent Form. This shall be done in accordance with local IRB regulations, as well as applicable State/Provincial and Federal Laws (see Appendix A, Section 1).

8.2 Observations and Measurements

Visit 1 (Day -21 to -14 – Screening)

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This is the initial screening visit, which will occur between 14 and 21 days prior to the first administration of study medication. The screening period may be extended to 4 weeks with permission of the sponsor. The following information will be obtained, or procedures performed, at this visit:

1. Signed informed consent will be obtained from each patient (see section 8.1, above).
2. Demographic data and medical/surgical history, including laboratory data for the previous 3 months, hemoglobin levels over the previous 6 months, and the number of transfusions and total number of units of blood transfused over the previous 12 months.
3. Weight in kilograms, sitting heart rate and blood pressure.
4. Complete physical examination.
5. 12-lead electrocardiogram (ECG).
6. Hematology, to include CBC (hemoglobin, hematocrit, RBC, WBC) with differential (segmented neutrophils, bands, eosinophils, basophils, lymphocytes, monocytes) and platelet count.
7. Blood Chemistries, to include blood glucose, BUN, uric acid, total bilirubin (conjugated and uncongugated), alkaline phosphatase, LDH, ALT (SGPT), AST (SGOT), creatinine, inorganic phosphorus, sodium, potassium, chloride, bicarbonate, calcium, albumin, and total protein.
8. Ferritin, vitamin B_{12}, and folate levels.
9. Urinalysis, to include pH, specific gravity, qualitative protein, glucose, ketones, bilirubin, urobilinogen, and microscopic examination of the sediment.
10. Flow cytometry (10 ml EDTA blood), to include RBC CD55 and CD59; PMN CD16, CD66, and CD55; monocyte CD14, CD64, and CD45; and platelet CD61 and CD55.
11. Throat culture for Neisseria.
12. Use of medications presently, and within the past thirty (30) days. This includes over the counter remedies and vitamin/herbal preparations.
13. Hepatitis B and C screen.
14. All women aged 55 years or less will have a serum β-HCG pregnancy evaluation to be performed at the local laboratory, unless surgically sterile (documented).
15. Vaccination against N. meningitidis (at least two weeks prior to first dose of study medication).

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Visit 2 (Day 0)

The EORTC QLQ-C30 must be administered **before** any study procedures or any evaluations by the study medical personnel.

The following procedures will be completed prior to initial dosing of study medication.

1. Record adverse events that have occurred since Screening.
2. Update concomitant medication record.
3. Sitting heart rate and blood pressure.
4. Hematology (see section 8.2, point 6 above).
5. Blood Chemistries (see section 8.2, point 7 above).
6. Coagulation profile, to include PT, PTT, INR.
7. Urinalysis (see section 8.2, point 9 above).
8. Reticulocyte count.
9. Serum haptoglobin.
10. Plasma (free) hemoglobin.
11. Ham Test.
12. Flow cytometry (see section 8.2, point 10 above).
13. EORTC QLQ-C30.
14. Self assessment of urine diary.
15. Throat culture for Neisseria.
16. Trough and peak samples for the determination of pharmacokinetic, pharmacodynamic, and C5b-9 parameters will be collected (see Appendix B).
17. Serum for HAHA (anti-h5G1.1-mAb) (see Appendix B).
18. Dosing – Once all of the above procedures/observations have been performed, the study medication may be administered. Patients should remain at the clinic for one hour following the end of the 30 minute infusion for observation.

9. DURING TREATMENT PROCEDURES

9.1 Observations and Measurements

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All study visits and study-associated procedures must occur within +/- 2 days of Visits 3 thru 13.

Visit 3 (Day 7)

The following observations/procedures will be performed:

1. Sitting heart rate and blood pressure.
2. The occurrence of adverse events will be noted.
3. Record the use/administration of concomitant medications.
4. Hematology (see section 8.2, point 6 above).
5. Blood Chemistries (see section 8.2, point 7 above).
6. Coagulation profile, to include PT, PTT, INR.
7. Reticulocyte count.
8. Flow cytometry (see section 8.2, point 10 above).
9. Self assessment of urine diary.
10. Dosing – Once all of the above procedures/observations have been performed, the study medication may be administered. Patients should remain at the clinic for one hour following the end of the 30 minute infusion for observation.

Visit 4 (Day 14)

The following observations/procedures will be performed:

1. Sitting heart rate and blood pressure.
2. The occurrence of adverse events will be noted.
3. Record the use/administration of concomitant medications.
4. Hematology (see section 8.2, point 6 above).
5. Blood Chemistries (see section 8.2, point 7 above).
6. Coagulation profile, to include PT, PTT, INR.
7. Reticulocyte count.
8. Serum haptoglobin.
9. Flow cytometry (see section 8.2, point 10 above).
10. Self assessment of urine diary.
11. Dosing – Once all of the above procedures/observations have been performed, the study medication may be administered. Patients should

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remain at the clinic for one hour following the end of the 30 minute infusion for observation.

Visits 5 (Day 21)

The following observations/procedures will be performed:

1. Sitting heart rate and blood pressure.
2. The occurrence of adverse events will be noted.
3. Record the use/administration of concomitant medications.
4. Hematology (see section 8.2, point 6 above).
5. Blood Chemistries (see section 8.2, point 7 above).
6. Coagulation profile, to include PT, PTT, INR.
7. Reticulocyte count.
8. Flow cytometry (see section 8.2, point 10 above).
9. Self assessment of urine diary.
10. Trough and peak samples for the determination of pharmacokinetic, pharmacodynamic, and C5b-9 parameters will be collected (see Appendix B).
11. Dosing – Once all of the above procedures/observations have been performed, the study medication may be administered. Patients should remain at the clinic for one hour following the end of the 30 minute infusion for observation.

Visits 6 (Day 28)

The EORTC QLQ-C30 must be administered **before** any study procedures or any evaluations by the study medical personnel.

The following observations/procedures will be performed:

1. Sitting heart rate and blood pressure.
2. The occurrence of adverse events will be noted.
3. Record the use/administration of concomitant medications.
4. Hematology (see section 8.2, point 6, above).
5. Blood Chemistries (see section 8.2, point 7, above).
6. Coagulation profile, to include PT, PTT, INR.
7. Urinalysis (see section 8.2, point 9, above).
8. Reticulocyte count.

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9. Serum haptoglobin.
10. Flow cytometry (see section 8.2, point 10 above).
11. EORTC QLQ-C30.
12. Self assessment of urine diary.
13. Throat culture for Neisseria.
14. Trough and peak samples for the determination of pharmacokinetic, pharmacodynamic, and C5b-9 parameters (see Appendix B).
15. Serum for HAHA (anti-h5G1.1-mAb) (see Appendix B).
16. Dosing – Once all of the above procedures/observations have been performed, the study medication may be administered. Patients should remain at the clinic for one hour following the end of the 30 minute infusion for observation.

Visits 7 (Day 35)

The following observations/procedures will be performed:

1. Sitting heart rate and blood pressure.
2. The occurrence of adverse events will be noted.
3. Record the use/administration of concomitant medications.
4. Hematology (see section 8.2, point 6 above).
5. Blood Chemistries (see section 8.2, point 7 above).
6. Reticulocyte count.
7. Flow cytometry (see section 8.2, point 10 above).
8. Interval samples for the determination of pharmacokinetic, pharmacodynamic, and C5b-9 parameters (see Appendix B).
9. Self assessment of urine diary.

Visits 8 (Day 42)

The following observations/procedures will be performed:

1. Sitting heart rate and blood pressure.
2. The occurrence of adverse events will be noted.
3. Record the use/administration of concomitant medications.
4. Hematology (see section 8.2, point 6 above).
5. Blood Chemistries (see section 8.2, point 7, above).
6. Reticulocyte count.

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7. Serum haptoglobin.
8. Flow cytometry (see section 8.2, point 10 above).
9. Trough samples for the determination of pharmacokinetic, pharmacodynamic, and C5b-9 parameters (see Appendix B).
10. Self assessment of urine diary.
11. Dosing – Once all of the above procedures/observations have been performed, the study medication may be administered. Patients should remain at the clinic for one hour following the end of the 30 minute infusion for observation.

Visits 9 (Day 56)

The EORTC QLQ-C30 must be administered **before** any study procedures or any evaluations by the study medical personnel.

The following observations/procedures will be performed:

1. Sitting heart rate and blood pressure.
2. The occurrence of adverse events will be noted.
3. Record the use/administration of concomitant medications.
4. Hematology (see section 8.2, point 6, above).
5. Blood Chemistries (see section 8.2, point 7, above).
6. Coagulation profile, to include PT, PTT, INR.
7. Urinalysis (see section 8.2, point 9, above).
8. Reticulocyte count.
9. Serum haptoglobin.
10. Flow cytometry (see section 8.2, point 10 above).
11. EORTC QLQ-C30.
12. Self assessment of urine diary.
13. Throat culture for Neisseria.
14. Serum for HAHA (anti-h5G1.1-mAb) (see Appendix B).
15. Dosing – Once all of the above procedures/observations have been performed, the study medication may be administered. Patients should remain at the clinic for one hour following the end of the 30 minute infusion for observation.

Visits 10 (Day 70)

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The following observations/procedures will be performed:

1. Sitting heart rate and blood pressure.
2. The occurrence of adverse events will be noted.
3. Record the use/administration of concomitant medications.
4. Hematology (see section 8.2, point 6 above).
5. Blood Chemistries (see section 8.2, point 7, above).
6. Reticulocyte count.
7. Serum haptoglobin.
8. Flow cytometry (see section 8.2, point 10 above).
9. Self assessment of urine diary.
10. Trough and peak samples for the determination of pharmacokinetic, pharmacodynamic, and C5b-9 parameters (see Appendix B).
11. Dosing – Once all of the above procedures/observations have been performed, the study medication may be administered. Patients should remain at the clinic for one hour following the end of the 30 minute infusion for observation.

10. POST-TREATMENT PROCEDURES

10.1 Observations and Measurements

All study visits and study-associated procedures must occur within +/- 2 days of the scheduled visit day for Visit 14.
Patients who drop from the study before Visit 14 should perform all evaluations listed under PT.

Visit 11 (Day 84)
The EORTC QLQ-C30 must be administered **before** any study procedures or any evaluations by the study medical personnel.

The following observations/procedures will be performed:

1. Complete physical examination.
2. Sitting heart rate and blood pressure.
3. 12-lead electrocardiogram (ECG).

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4. Hematology (see section 8.2, point 6, above).
5. Blood Chemistries (see section 8.2, point 7, above).
6. Ferritin, vitamin B_{12}, and folate levels.
7. Coagulation profile, to include PT, PTT, INR.
8. Urinalysis (see section 8.2, point 9, above).
9. Reticulocyte count.
10. Serum haptoglobin.
11. Plasma (free) hemoglobin.
12. Ham Test.
13. Flow cytometry (see section 8.2, point 10 above).
14. EORTC QLQ-C30.
15. Self assessment of urine diary.
16. Throat Culture for Neisseria.
17. Trough samples for the determination of pharmacokinetic, pharmacodynamic, and C5b-9 parameters (see Appendix B).
18. Serum for HAHA (anti-h5G1.1-mAb) (see Appendix B).
19. The occurrence of adverse events will be noted.
20. Record the use/administration of concomitant medications.
21. All women aged 55 years or less will have a serum β-HCG pregnancy evaluation to be performed at the local laboratory, unless surgically sterile (documented).

Patients who **are** participating in the extension study will also have the following observations/procedures performed:

1. Signed informed consent will be obtained from each patient (see section 8.1, above).
2. Weight in kilograms.
3. Dosing – Once all of the above procedures/observations have been performed, the study medication may be administered. Patients should remain at the clinic for one hour following the end of the 30 minute infusion for observation.

Patients who **are not** participating in the extension study will also have the following observations/procedures performed:

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1. Exchange transfusion **if** the PNH RBC's are >50% of the total RBC's by flow cytometry.

Visit 12 (Day 112)

The following observations/procedures will be performed for **all patients** whether or not participating in the extension study:

1. Sitting heart rate and blood pressure.
2. The occurrence of adverse events will be noted.
3. Record the use/administration of concomitant medications.

The following observations/procedures will be performed only on patients **participating** in the extension study:

1. Hematology (see section 8.2, point 6, above).
2. Blood Chemistries (see section 8.2, point 7, above).
3. Urinalysis (see section 8.2, point 9, above).
4. Dosing – Once all of the above procedures/observations have been performed, the study medication may be administered. Patients should remain at the clinic for one hour following the end of the 30 minute infusion for observation.

Premature Termination (PT) Visit

The EORTC QLQ-C30 must be administered **before** any study procedures or any evaluations by the study medical personnel.

The following observations/procedures will be performed:

1. Complete physical examination.
2. Sitting heart rate and blood pressure.
3. 12-lead electrocardiogram (ECG).
4. Hematology (see section 8.2, point 6, above).
5. Blood Chemistries (see section 8.2, point 7, above).
6. Ferritin, vitamin B_{12}, and folate levels.
7. Coagulation profile, to include PT, PTT, INR.

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8. Urinalysis (see section 8.2, point 9, above).
9. Reticulocyte count.
10. Serum haptoglobin.
11. Plasma (free) hemoglobin.
12. Ham Test.
13. Flow cytometry (see section 8.2, point 10 above).
14. EORTC QLQ-C30.
15. Self assessment of urine diary.
16. Throat Culture for Neisseria.
17. Trough samples for the determination of pharmacokinetic, pharmacodynamic, and C5b-9 parameters (See Appendix B).
18. Serum for HAHA (anti-h5G1.1-mAb) (see Appendix B).
19. The occurrence of adverse events will be noted.
20. Record the use/administration of concomitant medications.
21. All women aged 55 years or less will have a serum β-HCG pregnancy evaluation to be performed at the local laboratory, unless surgically sterile (documented).
22. Exchange transfusion **if** the PNH RBC's are >50% of the total RBC's by flow cytometry.

11. ADVERSE EVENTS

Information regarding adverse events (AEs) will be collected from the time the patient signs the informed consent form up to and including the visit 12.

11.1 Safety Definitions

Adverse Event: This is defined as any undesirable clinical experience occurring to a patient whether or not it is considered related to the investigational product.

Further, this undesirable experience should not be the condition that qualified the patient for participation in the study, i.e. PNH. A patient who experiences an exacerbation of PNH that is severe enough to warrant dropping from the study should be withdrawn from the study for lack of efficacy. Additionally, should the exacerbation be so severe as to require the patient's hospitalization, or

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prolongation of hospitalization, the event must be reported as a serious adverse event (SAE). See below for description of an SAE.

Undesirable experiences do however, include exacerbations of other pre-existing conditions, (i.e. other than PNH). That is, if a condition that was present prior to the start of the study occurs more frequently or with greater severity during the study, it constitutes an AE.

Detection

Adverse events may be volunteered spontaneously by the subject, be discovered as a result of general questioning by the Investigator, by physical examination, by ECG, and by laboratory. During all occasions of contact with the Investigator, subjects will be systematically observed and questioned regarding the occurrence of adverse events. Questions will be phrased so that they do not, by suggestion, "lead" the subject into giving information that is not valid.

Non-Treatment Emergent Adverse Events

When an AE occurs after written informed consent has been obtained, but prior to the first dose of study drug, or when the AE occurs more than 30 days after the last dose of study medication the AE will be considered a **non-treatment-emergent AE**. Only serious, non-treatment-emergent AEs that are related to study procedures will be collected.

If a patient dies within 30 days of the last dose of study medication however, the death will be reported as a SAE, regardless of causality.

Treatment Emergent Adverse Events

When an AE occurs between the time the patient receives his or her first dose of study medication and a) his or her exit from the study or b) 30 days after the last dose of study medication, the AE will be considered a **treatment-emergent AE**, except as noted above.

Immediately Reportable Adverse Event (IRAE): This is an AE that must be reported to Alexion Pharmaceuticals within 24 hours of the study center being

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informed of the AE. Immediately reportable AEs include all serious AEs, as listed below, **and** AEs that result in a patient's withdrawal from the study.

Serious Adverse Event: As provided by the ICH criteria, any AE that:

- results in death;
- is life threatening;

(Note: The term "life threatening" refers to an AE that, as it occurs, puts the patient at immediate risk of death. It does not refer to an AE that hypothetically might have caused death if it were more severe.)

- results in hospitalization or prolongation of current hospitalization (not including hospitalization for a pre-existing condition that has not increased in severity or frequency from the patient's underlying medical condition prior to entry into the study);
- results in persistent or significant disability/incapacity;
- is a congenital anomaly/ birth defect in the offspring of a patient; or
- is judged to be medically significant.

(Note: A medically significant AE is a medical event that may not be immediately life-threatening or result in death or hospitalization but may jeopardize the patient or require intervention to prevent one of the outcomes listed above. Medical and scientific judgment should be exercised in deciding whether other AEs appropriately meet these criteria and are immediately reportable to the Sponsor or designee.)

When completing the appropriate forms for reporting the AE, the investigator will be asked to assess the AE as follows:

Severity of Adverse Event: Refers to the extent to which an AE affects the patient's daily activities. Severity will be categorized according to the following criteria:

Mild:	Normal activities unaltered or annoying, but tolerable.
Moderate:	Normal activities altered or require intervention.
Severe:	Unable to undertake normal activities or incapacitated.

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Causality of Adverse Events: Refers to the relationship of the AE to study drug.

Causality will be assessed by the investigator and categorized according to the following criteria:

Unlikely: There is no medical evidence to suggest that the AE is related to study medication usage, or there is a more probable medical explanation.

Possible: There is medical evidence to suggest that the AE is related to study drug usage. However, other medical explanations cannot be excluded as a possible cause.

Probable: There is strong medical evidence to suggest that the AE is related to study drug usage.

11.2 Reporting Adverse Events

When an AE is suspected, all relevant evaluations will be performed and appropriate treatment provided. For non-serious AEs that continue past the last study visit, the subject will be contacted by telephone seven days following their last visit to determine if the event has resolved, is ongoing or has worsened. If the severity and frequency of the AE(s) has not changed, it will be marked as 'Ongoing" on the CRF. If the event has worsened the subject will remain under medical supervision until the investigator and the Sponsor's medical monitor and/or clinical safety director deem the AE to be resolved, stabilized, or no longer serious enough to warrant follow-up. For SAEs that continue past the last study visit, the subject will be contacted by telephone thirty days following their last visit to determine if the event has resolved, is ongoing or has worsened. If the event has worsened the subject will remain under medical supervision until the investigator and the Sponsor's medical monitor and/or clinical safety director deem the SAE to be resolved, stabilized, or no longer serious enough to warrant follow-up.

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All AEs, both serious and non-serious will be recorded on the Adverse Event Log of the CRF.

All Adverse Events

Serious non-treatment-emergent AEs related to study procedures and all treatment-emergent AEs (serious and non-serious) will be reported on the appropriate tabs on the CRF.

Serious Adverse Events

Information regarding serious AEs will be recorded on the appropriate tabs of the CRF.

In addition, the investigator or designee must complete the IRAE section of the CRF, as well as the Concomitant Medications and Demographics sections. These forms must be completed and Alexion Pharmaceuticals notified within 24 hours of the study center being informed of the SAE.

Withdrawals Due to Adverse Events

When study medication is discontinued due to an AE, or the patient withdraws from the study due to an AE, whether or not the event was related to study medication, the investigator or designee must complete the Adverse Event Log CRF, and the Patient Accountability, Concomitant Medications and Demographics sections of the CRF. The investigator must Alexion Pharmaceuticals within 24 hours of the study center being informed of the AE.

11.3 Exposure in Utero

If it becomes known during the administration of study medication that a patient is pregnant, the study medication will be stopped. In addition, the study medication will be discontinued for any woman who becomes pregnant during the extension study. Alexion Pharmaceuticals should be notified, and will supply the investigator with a copy of an "Exposure in utero" form. The patient should be followed until the outcome of the pregnancy is known, at which time the form should be completed

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and returned to the Sponsor. If additional follow-up is required, the investigator will be informed.

11.4 Adverse Event Reporting Contacts

For Immediately Reportable Adverse Events (IRAEs), the investigator should contact Alexion Pharmaceuticals at the following numbers:

North America
Telephone Number: 011-203-271-8215 or 011-203-272-2596
Fax Number: 011-203-271-8196

12. CRITERIA FOR THE EVALUATION OF STUDY RESULTS

12. 1 Criteria for the Evaluation of Efficacy

- As this is a pilot study, there is no formal primary efficacy endpoint.

The secondary endpoints comprise changes in:

- Number of units of PRBCs transfused.
- Time to first transfusion of PRBCs.
- Change from baseline hemoglobin and hematocrit.
- Change from baseline of indicators of hemolysis including reticulocyte count, haptoglobins, LDH and bilirubin.
- Number of paroxysms and time to first paroxysm (defined as urine of color grade $\geq$ 6; See Appendix D).
- Reticulocyte count.
- Serum haptoglobin
- Change from baseline EORTC QLQ-C30
- The extent and duration of complement inhibition (pharmacodynamics).

The exploratory endpoints consist of changes in:

- Platelet and neutrophil count

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- Change in the size of the GPI-deficient (PNH) clone, assessed by flow cytometry (including RBC's, PMN's, monocytes and platelets).
- Ham test
- Plasma (free) hemoglobin

12.2 Criteria for the Evaluation of Safety

Safety will be measured by collecting data on adverse events and laboratory values. A complete laboratory evaluation (hematology, blood chemistries and urinalysis) will be done at screening, pre-dose, during the active treatment period (days 0-84), and at study termination (day 84). Specific attention will be given to the occurrence of infection.

12.3 Evaluation of Pharmacokinetic and Pharmacodynamic Data

The trough concentrations of Eculizumab collected from patients at Visits 2 through 12 will be used to calculate the pharmacokinetic profile of the drug in patients with PNH. Similarly, the pharmacodynamics of Eculizumab as regards the degree and duration of the blockade of C5 will also be determined. Data will be compared to a preliminary pharmacokinetic/pharmacodynamic model of Eculizumab, which has been generated from other Phase I data. The analysis has been, and will be, performed with the USC-PACK suite of PK analysis tools (using both the iterative two- stage parametric Bayesian (IT2B) as well as the Non-parametric Expectation Maximization (NPEM) methods).

12.4 Evaluation in Case of Discontinued Treatment

As all patients who receive any amount of study medication are eligible for safety and intention to treat analyses, all observations and measurements, as outlined in Sections 8, 9, and 10, above, should be performed. If a patient drops out of the study all procedures listed under PT Visit should be performed at the termination visit.

12.5 Removal of Patients from Study or Analysis

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Patients may be removed from the study because of serious adverse events, as well as conditions or intercurrent illnesses that preclude compliance with the protocol from the standpoint of a patient's safety or well-being. Patients may also be removed because of translocation to a different geographical area or otherwise being lost to follow-up.

If a patient is withdrawn from the study after receiving any amount of study medication, he or she will be considered as having prematurely terminated the study and will not be replaced. All such patients will be eligible for the safety and intention-to-treat analyses.

12.6. Evaluation of Data for the Safety and Protection of Subjects

Due to the small size and short duration of this study, no data and safety monitoring board will be used.

12.7 Stopping Rules

None

13. DATA PROCESSING AND ANALYSIS

13.1 Sample Size Estimation

Not applicable, as this is a pilot study.

13.2 Statistical and Analytical Plan

Demographics and Baseline Data

Demographics data and other baseline data will be examined and presented as a single group. Summary statistics such as means, medians, and standard deviations for continuous data, and percentages for categorical data will be used to present distributions of patient characteristics.

Efficacy Assessments

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Due to the small sample size, no formal statistical analyses will be performed.

Summary statistics (frequency and percentage for categorical endpoint, mean, median, standard deviation, and range for continuous variable) will be presented for pretreatment vs. post-treatment values for all secondary and exploratory endpoints. The difference in the incidence of outcome among treatment groups will be tested using Fisher's Exact test, or Chi-square statistics. The difference in the mean, median or scores of the outcome measures among the treatment groups will be tested using T-test, Analysis of Variance (or Wilcoxon rank sum test and Kruskal-Wallis test for data with non-normal distribution).

Safety

Any patient who receives any amount of study medication and has post baseline safety data is included for safety evaluation.

Safety will be assessed by examination of adverse events, clinical laboratory data, and vital signs. Safety parameters will be assessed during the study period including the follow-up period.

Adverse events (both patient and event counts) will be tabulated by severity and, for each severity, by investigator-assessed relationship to study drug. In addition, adverse events related to the occurrence of infection will be tabulated separately.

Hematology, blood chemistry, and urinalysis data will be analyzed using distribution shift tables. Laboratory values will be categorized as normal, abnormally low, or abnormally high at baseline and at post-baseline assessments. In addition, mean changes from pre-infusion to post-dose assessments will be summarized by treatment group using the mean, median, standard deviation, and range.

Data from the pharmacodynamic and complement assays (PD, C5b-9) will be presented using summary statistics such as mean, median, standard deviation and range at each post-baseline assessment. Vital signs (blood pressure, heart

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rate, and temperature) will be summarized by the mean change from baseline to post-baseline assessments. In addition the median, standard deviation, and range at each post-baseline assessment will be presented.

Pharmacokinetic Parameters

Since only trough samples will be used, maximum plasma concentration (Cmax), time to maximum concentration (Tmax), terminal rate constant and half-life, area under the serum concentration versus time curve (AUC), and apparent column distribution and apparent clearance will not be calculated.

13.3 Interim Analysis

An interim analysis is not foreseen.

13.4 Use of Data and Publication

Please refer to the terms outlined in your study specific Clinical Study Agreement or Master Clinical Study Agreement.

14. ESTIMATED DURATION OF THE STUDY

The estimated duration of the study, given a three month enrollment period, is 7 months.

15. PREMATURE TERMINATION OF THE STUDY

Please refer to the terms outlined in your study specific Clinical Study Agreement or Master Clinical Study Agreement.

16. PHARMACOKINETICS AND PHARMACODYNAMICS

Assays for anti-human antibodies (HAHA) and C5b-9 will be performed at the laboratories of Alexion Pharmaceuticals. Pharmacokinetic and pharmacodynamic assays will also be performed at Alexion Pharmaceuticals.

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Samples at the times specified for assays in Figure 1 should be taken as detailed in Appendix B, and labeled with the labels provided.

Shipment of Frozen Samples

All frozen samples for h5G1.1-mAb neutralizing factor (HAHA), C5b-9, pharmacodynamics and pharmacokinetics will be shipped to

Michael Straub
Alexion Pharmaceuticals, Inc.
352 Knotter Drive
Cheshire, CT 06410.

Please refer to the shipping instructions as detailed in Appendix C, and in the Clinical Laboratories Manual and Clinical laboratories summary chart.

17. PROJECT MONITORS

The Clinical Project Director for this study will be:

Christopher F. Mojcik M.D., Ph.D.
Alexion Pharmaceuticals, Inc.
352 Knotter Drive
Cheshire, CT 06410
(203) 272-2596 (office)
(203) 784-4333 (pager)

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18. STUDY TIMETABLE

Projected starting date: 05/01/02
Projected number of subjects entered: 10
Projected number of subjects valid for efficacy analyses: 10
Projected completion date: 11/31/02

The following documents must be submitted to Alexion Pharmaceuticals, Inc. prior to the start of the study:

- Curriculum Vitae (Principal and Sub-Investigators)
- Institutional Review Board (IRB) Approval
- Copy of the IRB Approved Informed Consent Form
- Signed copy of protocol
- Signed copy of the FDA 1572 Form (USA IND Studies)
- Qualified Investigator Undertaking (Canadian sites only)
- Research Ethics Board Attestation (Canadian sites only)

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APPENDIX A

Ethical and Regulatory Considerations

The following must be observed to comply with regulatory agencies' regulations for the conduct and monitoring of clinical investigations; they also represent sound clinical research practice:

A. Informed Consent

The principles of informed consent are described in ICH Guideline "Good Clinical Practice: Consolidated Guideline", and other federal regulatory agency documents as listed in section 4. They must be followed to comply with Food and Drug Administration and other local regulatory agencies' regulations for the conduct and monitoring of clinical investigations. In seeking informed consent, the following information shall be provided in a language understandable to the subject.

1. The following are the basic elements of informed consent, which should be provided to each subject:

   a. A statement that the study involves research, an explanation of the purposes of the research and the expected duration of the subject's participation, a description of the procedures to be followed, and identification of any procedures which are experimental.
   b. A description of any reasonably foreseeable risks or discomforts to the subject.
   c. A description of any benefits to the subject or to others which may reasonably be expected from the research.
   d. A disclosure of appropriate alternative procedures or courses of treatment, if any, that might be advantageous to the subject.
   e. A statement describing the extent, if any, to which confidentiality of records identifying the subject will be maintained and that notes the possibility that the regulatory agencymay inspect the records.
   f. For research involving more than minimal risk, an explanation as to whether any compensation and an explanation as to whether any medical treatments are available if injury occurs and, if so, what they consist of, or where further information may be obtained.
   g. An explanation of whom to contact for answers to pertinent questions about the research and research subjects' right, and whom to contact in the event of a research-related injury to the subject.
   i. A statement that participation is voluntary, that refusal to participate will involve no penalty or loss of benefits to which the subject is otherwise entitled, and that the subject may discontinue participation at any time without penalty or loss of benefits to which the subject is otherwise entitled.

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2. When appropriate, one or more of the following elements of information shall also be provided to each subject:

   a. A statement that the particular treatment or procedure may involve risks to the subject (or to the embryo or fetus, if the subject is or may become pregnant) which are currently unforeseeable.
   b. Anticipated circumstances under which the subject's participation may be terminated by the investigator without regard to the subject's consent.
   c. Any additional costs to the subject that may result from participation in the research.
   d. The consequences of a subject's decision to withdraw from the research and procedures for orderly termination of participation by the subject.
   e. A statement that significant new findings developed during the course of the research which may relate to the subject's willingness to continue participation will be provided to the subject.
   f. The approximate number of subjects involved in the study.

A subject (or the subject's legally authorized representative) must give his/her written consent to participate in the study. This consent must be witnessed, dated and retained by the investigator as part of the study records. A copy of the consent form must be given to the subject.

If Experimental Subject's Bill of Rights is applicable (as of January 1988, only applicable in the State of California), this form must also be prepared and signed by each subject and retained as part of the required study records. A copy of the Bill of Rights must be given to the subject.

A copy of the proposed consent form must be submitted to the Institutional Review Board together with the protocol for approval. A copy of the IRB approved consent form must be available for inspection by the Alexion Study Monitor/Research Associate (or monitors/associates contracted by Alexion), and evidence of an IRB approved consent form may be required prior to shipment of drug supplies to the investigator. Each subject's signed informed consent form must be kept on file by the investigator for regulatory agency/Monitor inspection at any time.

B. Institutional Review

The protocol and informed consent form for this study must be approved in writing by the appropriate Institutional Review Board, as defined by ICH Guideline "Good Clinical Practice: Consolidated Guideline", and other federal regulatory agency documents as listed in section 4)
A copy of the letter of approval from the Board (Research Ethics Board Attestation in Canada), which should contain specific identification of the documents approved, must be received by the Study Monitor/Research Associate at Alexion Pharmaceuticals or its designee prior to shipment of drug supplies to the investigator.

All changes to approved protocols, including amendments and change of principal investigator, must be done in writing and be approved by the Board prior to implementation, and documentation of this approval provided to the Study Monitor/Research Associate. If there is any amendment to protect subjects from an

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immediate hazard, it may be implemented immediately, and must be reported to the IRB in writing within 10 working days. The IRB still must approve the amendment. Records of the Institutional Review Board review and approval of all documents pertaining to this study must be kept on file by the investigator and are subject to Alexion or regulatory agency inspection at any time during the study.

Periodic status reports must be submitted to the IRB, at least yearly, as well as notification of completion of the study. A copy of these reports should be sent to the Study Monitor/Research Associate. The investigator must maintain an accurate and complete record of all reports and documents submitted to the IRB.

C. Recording of Data

Data for individual subjects are to be recorded on remote data entry screens, as designed by Alexion Pharmaceuticals.

A set of RDE screens must be completed for each subject enrolled, including those removed from the study. If a subject is removed from study, the reason for removal must be noted on the appropriate RDE screen by the investigator or his/her designee. The principal investigator must review all screens and will indicate, by his or her signature at the end of the trial, the veracity of the information contained therein.

RDE screens must be kept current to reflect subject status at each phase during the course of the study. Subjects are not to be identified on the screens by name; appropriate coded identification and patient initials must be used. The investigator/designee must keep a separate log of patient names and addresses. If requested during a regulatory agency inspection, this log may be shown to the regulatory agency investigator but no copy should be provided.

The RDE data, appropriate source documentation and copies of test results must be available at all times for inspection by the Study Monitor/Research Associates and regulatory agency.

D General Information

1. Study records including case report forms, signed FDA 1572, Qualified Investigator Undertaking in Canada, originals of test results, signed informed consent forms, Institutional Review Committee approval letters (Research Ethics Board Attestation in Canada), copies of documents submitted to the IRB, and other documents pertaining to the conduct of the study must be kept on file by the investigator. If the investigator wishes to assign the files to someone else or remove them to another location he should inform the Alexion Pharmaceuticals monitor in writing about this change. Study records are subject to FDA inspection at any time. Study records must be maintained for a minimum of two years following written notification by Alexion Pharmaceuticals of either regulatory agency marketing authorization or discontinuance of the IND.

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2. The Study Monitor/Research Associate must be acquainted with the investigator and other key people involved in the study. Monitoring visits will be scheduled periodically and regularly. It is essential that the investigator and the designated study coordinator set aside a sufficient amount of time for these visits to permit an adequate review of the study's progress and of completed case reports.

3. If there is a change or addition of sub-investigator, the FDA 1572 form and Qualified Investigatory Undertaking in Canada must be promptly amended.

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APPENDIX B

PROCEDURE FOR COLLECTING STUDY C02-001 CLINICAL RESEARCH SAMPLES

PD – Pharmacodynamic – Hemolysis, PK – Pharmacokinetic, and HAHA – Human Anti-Human Antibody Assays

1. **Serum** is used in these assays.
2. Blood is drawn into tubes that **do not** contain anticoagulant.
3. Blood is allowed to clot at room temperature for 30 minutes to 1 hour.
4. Centrifuge the tubes.
5. Remove serum and place at least 1ml into labeled tube(s). **Please note, for visit 2, 5 tubes with at least 1 ml of serum per tube is required for PK.**
6. The serum containing tubes are stored at –70°C until they are shipped to Alexion Pharmaceuticals, Inc.

PD – Pharmacodynamic – C5b-9 Complement Assay

1. **Plasma** is used in this assay.
2. Blood is drawn into EDTA-containing tubes.
3. Centrifuge the tubes immediately (within 2 hours).
4. Remove the plasma and place at least 1 ml into a labeled vial.
5. The serum containing tubes are stored at –70°C until they are shipped to Alexion Pharmaceuticals, Inc.

Note: The Serum and Plasma may be maintained up to 4 hours at 2-8°C (refrigerated or on wet ice) prior to final storage at –70°C.

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APPENDIX C
Study C02-001 Clinical Research Sample Shipping Instructions

PK	Pharmacokinetics
PD	Pharmacodynamics – Hemolysis
C5b-9	Pharmacodynamics – C5b-9 Complement Assay
HAHA	Human Anti-Human Antibody – Neutralizing Antibody

Samples Labeled PK, PD, HAHA, and C5b-9

1. Ship by patient, once the patient has completed the study, to Alexion Pharmaceuticals, Inc.
2. Pack all samples frozen on dry ice

Michael Straub

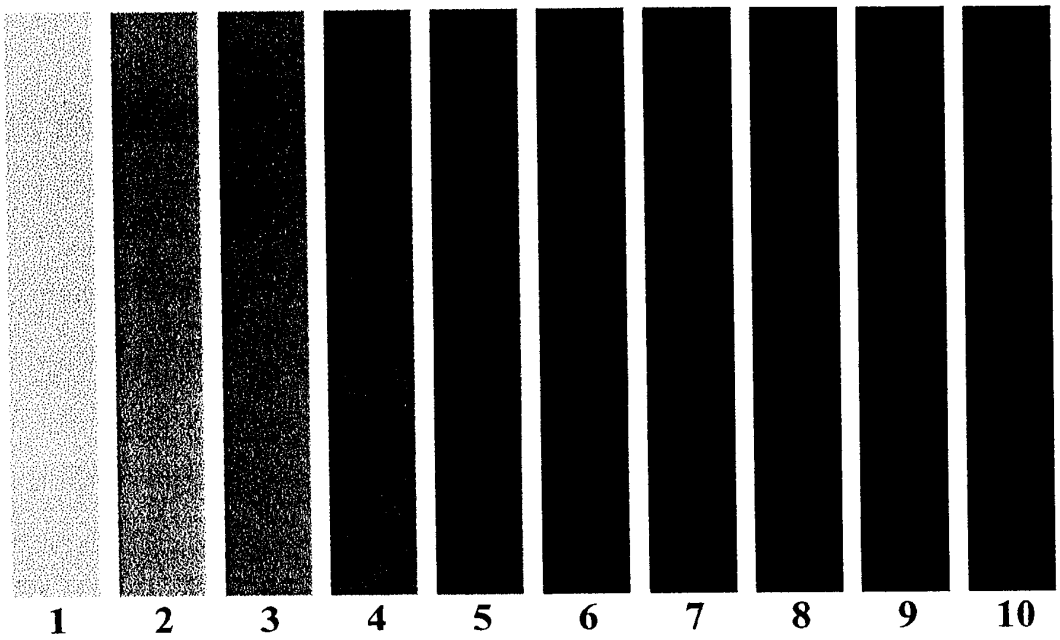
Alexion Pharmaceuticals, Inc.

352 Knotter Drive
Cheshire, CT 06410

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APPENDIX D

Paroxysm Grading



Colour of urine first time in the morning

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CONFIDENTIAL DISCLOSURE AGREEMENT

CONFIDENTIAL DISCLOSURE AGREEMENT (the "Agreement"), dated as of January 11, 2002, between Alexion Pharmaceuticals, Inc., with offices at 352 Knotter Drive, Cheshire Connecticut 06410 ("Alexion"), and Dr. Peter Hillmen, with offices at Haematological Malignancy Diagnostic Service, Algernon Firth Building, Leeds General Infirmary, Great George Street, Leeds, UK LS1 3EX (the "Receiving Party").

WHEREAS, Alexion possesses certain valuable and confidential information relating to the research and development, clinical development, therapeutic opportunities (including PNH) and business matters involving its proprietary antibody candidate referred to as h5G1.1-mAb (the "Information"); and

WHEREAS, Alexion and the Receiving Party wish to explore the possibility of entering into certain arrangements relating to the Information;

NOW, THEREFORE, in consideration of the mutual promises contained herein, Alexion and the Receiving Party hereby agree as follows:

1. Alexion shall disclose such of the Information as it deems necessary and the Receiving Party shall accept and hold the Information in confidence in accordance with the provisions of Paragraph 2 hereof.

2. The Receiving Party agrees not to (1) disclose to any third party any or all of the Information, or permit any third party to have access to such Information, or (2) use such Information for any purpose other than to evaluate the Information in connection with discussions between the parties. In addition, the Receiving Party agrees to disclose the Information only to those of its employees who shall reasonably need to know the Information in order to evaluate the Information and who are bound to terms of confidentiality that are substantially as protective as the terms hereof. However, such obligations assumed by the Receiving Party shall not apply to any of the following:

   a. Information which is in the public domain at the time of disclosure hereunder or which subsequently comes within the public domain through no fault of or action by the Receiving Party; and

   b. Information which is in the possession of the Receiving Party at the time of disclosure by Alexion or which is independently discovered, after the date hereof, by the Receiving Party without the aid, application or use of the Information, in each such case as evidenced by written records; and

   c. Information which is obtained, after the date hereof, by the Receiving Party from any third party that is lawfully in possession of such Information and not in violation of any contractual or legal obligation with respect to such Information.

3. This Agreement, including the confidentiality and limited use obligations hereunder, shall remain in effect for a period of seven (7) years from the date of disclosure of the Information.

4. The Receiving Party shall return to Alexion, upon request, any and all written documents and samples of any materials provided by Alexion hereunder, except that the Receiving Party may retain one copy for its Legal Division for record keeping purposes only.

5. No right or license, either express or implied, with respect to the Information is granted hereunder by Alexion to the Receiving Party. The disclosure of the Information by Alexion to the Receiving Party shall not result in any obligation on the part of either party to enter into any future agreement relating to the Information or to undertake any other obligation not set forth in a written agreement signed by the parties hereto.

6. This Agreement contains all the representations and agreements between the parties relating to the Information and any representation, promise or condition concerning the same which is not contained herein or in a superseding written agreement referring to this Agreement shall not be binding on either party hereto.

7. This Agreement shall be governed by and construed in accordance with the laws of the State of Connecticut (as the same would be applied to agreements entered into in Connecticut by two residents thereof), and each party agrees to the jurisdiction of the State and federal courts of Connecticut.

IN WITNESS WHEREOF, the Receiving Party and Alexion have caused this Agreement to be executed by their respective duly authorized representatives as of the date first above written.

ALEXION PHARMACEUTICALS, INC.

By: _____
Name: T. Dubin
Title: VP + General Counsel

_____
Dr. Peter Hillmen

CDA _ Dr. Hillmen 1-10-02

Form of Indemnity for Clinical Studies

To: The Leeds Teaching Hospitals NHS Trust, Beckett Street, LEEDS LS9 7TF ("the Authority")

From: Alexion Pharmaceuticals, Inc. ("the Sponsor")

Re: Clinical Study No C02-001 with Eculizumab

1 It is proposed that the Authority should agree to participate in the above sponsored study ("the Study") involving patients of the Authority ("the Subjects") to be conducted by Dr. Peter Hillmen (the "Investigators") in accordance with the protocol annexed, as amended from time to time with the agreement of the Sponsor and the Investigator ("the Protocol"). The Sponsor confirms that it is a term of its agreement with the Investigators that the Investigators shall obtain all necessary approvals of the applicable Local Research Ethics Committee and shall resolve with the Authority any issues of a revenue nature.

2 The Authority agrees to participate by allowing the Study to be undertaken on its premises utilising such facilities, personnel and equipment as the Investigators may reasonably need for the purpose of the Study.

3 In consideration of such participation by the Authority, and subject to paragraph 4 below, the Sponsor indemnifies and holds harmless the Authority and its employees and agents against all claims and proceedings (to include any settlements or ex-gratia payments made with the consent of the parties hereto and reasonable legal and expert costs and expenses) made or brought (whether successfully or otherwise):

3.1 by or on behalf of Subjects taking part in the Study (or their dependants) against the Authority or any of its employees or agents for personal injury (including death) to Subjects arising out of or relating to the administration of the product(s) under investigation or any clinical intervention or procedure provided for or required by the Protocol to which the Subjects would not have been exposed but for their participation in the Study.

3.2 by the Authority, its employees or agents or by or on behalf of a Subject for declaration concerning the treatment of a Subject who has suffered such personal injury.

4 The above indemnity by the Sponsor shall not apply to any such claim or proceeding:

4.1 to the extent that such personal injury (including death) is caused by the negligent or wrongful acts or omissions or breach of statutory duty of the Authority, employees or agents;

4.2 to the extent that such personal injury (including death) is caused by the failure of the Authority, its employees, or agents to conduct the Studies in accordance with the Protocol;

4.3 unless as soon as reasonably practicable following receipt of notice of such claim or proceeding, the Authority shall have notified the Sponsor in writing of it and

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shall, upon the Sponsor's request, and at the Sponsor's cost, have permitted the Sponsor to have full care and control of the claim or proceeding using legal representation of its own choosing;

4.4 if the Authority, its employees, or agents shall have made any admission in respect of such claim or proceeding or taken any action relating to such claim or proceeding prejudicial to the defence of it without the written consent of the Sponsor such consent not to be unreasonably withheld provided that this condition shall not be treated as breached by any statement properly made by the Authority, its employees or agents in connection with the operation of the Authority's internal complaint procedures, accident reporting procedures or disciplinary procedures or where such statement is required by law.

5 The Sponsor shall keep the Authority and its legal advisers fully informed of the progress of any such claim or proceeding, will consult fully with the Authority on the nature of any defence to be advanced and will not settle any such claim or proceeding without the written approval of the Authority (such approval not to be unreasonably withheld).

6 Without prejudice to the provisions of paragraph 4.3 above, the Authority will use its reasonable endeavours to inform the Sponsor promptly of any circumstances reasonably thought likely to give rise to any such claim or proceeding of which it is directly aware and shall keep the Sponsor reasonably informed of developments in relation to any such claim or proceeding even where the Authority decides not to make a claim under his indemnity. Likewise, the Sponsor shall use its reasonable endeavours to inform the Authority of any such circumstances and shall keep the Authority reasonably informed of developments in relation to any such claim or proceeding made or brought against the Sponsor alone.

7 The Authority and the Sponsor will each give to the other such help as may reasonably be required for the efficient conduct and prompt handling of any claim or proceeding by or on behalf of Subjects (or their dependants) or concerning such a declaration as is referred to in paragraph 3.2 above.

8 Without prejudice to the foregoing if injury is suffered by a Subject while participating in the Study, the Sponsor agrees to operate in good faith the Guidelines published in 1991 by The Association of the British Pharmaceutical Industry and entitled "Clinical Trial Compensation Guidelines " (where the Subject is a patient) and the Guidelines published in 1988 by the same Association and entitled "Guidelines for Medical Experiments in non patient Human Volunteers" (where the Subject is not a patient) and shall request the Investigator to make clear to the Subjects that the Study is being conducted subject to the applicable Association Guidelines.

9 For the purpose of this indemnity, the expression "agents" shall be deemed to include without limitation any nurse or other health professional providing services to the Authority under a contract for services or otherwise and any person carrying out work for the Authority under such a contract connected with such of the Authority's facilities and equipment as are made available for the Study under paragraph 2 above.

10 This indemnity shall be governed by and construed in accordance with English law.

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SIGNED on behalf of The Leeds Teaching Hospitals NHS Trust

Signature: .....

Date of Signature: 15/07/03 .....

15 July 2003

Position: **Associate Director of R&D**

DR. D. R. NORFOLK
ASSOCIATE R & D DIRECTOR
LEEDS TEACHING HOSPITALS NHS TRUST

SIGNED on behalf of the Company

Signature: .....

Date of signature: 7-3-03 .....

Position: VP TGC .....

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CLINICAL TRIAL SERVICES - DURHAM
ALEXION C02-001

DRUG DESTRUCTION FORM

SITE: 010

PHARMACY CONTACT: Caroline Bedford

PHONE: +44 (0) 113 392 2459

FAX:+44 (0) 113 392 8279

DATE: 3/Apr /2003.

Vial Numbers (Record one Vial # per line)	Empty/Partial/Unused	Date Destroyed	Site Initials/Date
20001	Partial	3/4/03	3/4/03
20002	Empty		
20003	Empty		
20004	Empty		
20005	Empty.		
20006	Partial		
20013	Empty		
20014	Empty.		
20015	Partial		
20029	Empty		

All entries must be made in black ink. Any errors should be crossed out with a single stroke, initialed, and dated. The use of white out, correction tape or scribbling out errors is not permitted. Once complete and verified by the study monitor, please fax a copy of this form to CTS (919) 479-2633.

Method of Destruction: Incineration

PLEASE PLACE COPY OF YOUR STANDARD OPERATING PROCEDURES ON DRUG DESTRUCTION IN THIS BINDER.

352 Knotter Drive ▲ Cheshire, CT ▲ 06410 ▲ Phone 203-272-2596 ▲ Fax 203-271-8196 ▲ www.alxn.com

Alexion Pharmaceuticals, Inc.
EXHIBIT II, ATTACHED TO MASTER CLINICAL STUDY AGREEMENT, FOR SPECIFIC STUDY SCOPE, BUDGET & PAYMENT SCHEDULE WITH ST. GEORGE'S HOSPITAL MEDICAL SCHOOL

Study Specifications

Upon signature, this Exhibit will be incorporated into and made part of the Master Clinical Study Agreement between Alexion Pharmaceuticals, Inc. ("Alexion") and St. George's Hospital Medical School ("Institution") dated May 1, 2002.

1. Product/Compound: h5G1.1-mAb ("Study Drug")

2. Protocol # and Title: C02-001, entitled: "An Open-Label Pilot Study of the Effect of Eculizumab (h5G1.1-mAb) on Patients with Transfusion Dependent, Hemolytic, Paroxymal Nocturnal Hemoglobinuria" ("Study").

3. Principal Investigator: Judith Marsh, MD

4. Contract Research Organization (if applicable, if not put N/A): Kendle

5. Scope:

   The Study will begin following IRB approval and receipt by Alexion of relevant Study documents. The Study will begin around May 1, 2002 and the anticipated completion date is December 31, 2003. Such dates may be modified by written agreement executed by Principal Investigator and authorized representatives of both Institution and Alexion.

6. Subjects:

   The anticipated number of completed and valid subjects in the Study shall be enrolled within the time frame described above, and according to the criteria listed in the Protocol.





7.2 Definitions:

A "Qualified Study Subject" is a research subject who has been randomized and has met the entrance criteria as defined in the Protocol or who is given written permission by Alexion to enter the Study.

A "Protocol Compliant Study Subject" is a Qualified Study Subject who has met the minimum attendance and compliance standards in the Protocol for evaluation of effectiveness of the Study Drug, and has completed the Study.

A "Non-Protocol Compliant Study Subject" is a Qualified Study Subject that violates the Study procedures, such as, insufficient clinic attendance, poor protocol compliance or other protocol violations, as defined in the Protocol, but the subject completes the Study.

An "Early-Terminated Study Subject" is a Qualified Study Subject who has been withdrawn from the Study because of: lack of efficacy, adverse event, lost to follow-up, or voluntary patient withdrawal. Additional follow-up may be required for these patients to meet FDA regulations.

A "Screen Failure Study Subject" is a study subject who meets all entrance criteria and consents to enroll in the study but fails to randomize due to, e.g., unacceptable laboratory values at the screening visit.

A "Non-Qualified Study Subject" is a research subject who has not met the entrance criteria as defined in the Protocol.







8. Provisions of Master Clinical Study Agreement

The Principal Investigator agrees to be bound by the terms of the Master Clinical Study Agreement between Alexion and Institution dated, May 1, 2002. Without limiting the foregoing, the Principal Investigator agrees to the intellectual property provisions of Section 2 of the Master Clinical Study Agreement and the confidentiality provisions of Section 7 of the Master Clinical Study Agreement.

9. Notices

All communications and notices required or permitted under this Agreement shall be deemed sufficiently served if hand delivered or served by certified mail, postage prepaid, addressed as follows:

TO ALEXION:

Thomas I. H. Dubin
Vice President and General Counsel
Alexion Pharmaceuticals, Inc.
352 Knotter Drive
Cheshire, CT 06410

TO ALEXION:
Thomas I. H. Dubin
Vice President and General Counsel
Alexion Pharmaceuticals, Inc.
352 Knotter Drive
Cheshire, CT 06410

TO PRINCIPAL INVESTIGATOR:
Judith Marsh, MD
St. George's Hospital
Blackshaw Road
London SW17 0QT

TO INSTITUTION:
Mr. Frank Shore, Director of Finance
St. George's Hospital Medical School
Ground Floor Hunter Wing
Cranmer Terrace
Tooting, London SW17 0RE

ACCEPTED BY:

Alexion Pharmaceuticals, Inc.
By: _____
Thomas I. H. Dubin
Vice President and General Counsel

St. George's Hospital Medical School
By: _____
Frank Shore
Director of Finance

The undersigned, as Principal Investigator, hereby agrees to accept responsibility for performance of the Protocol described herein, acknowledges having reviewed this Agreement and agrees to be bound by the terms and conditions stated herein and in the Master Clinical Study Agreement dated, May 1, 2002, which is incorporated herein by reference.

PRINCIPAL INVESTIGATOR

_____
Judith Marsh, MD
5/2/03
Date

FORM OF INDEMNITY FOR CLINICAL STUDIES

To: Alexion Pharmaceuticals, Inc., 352 Knotter Drive, Cheshire, CT 06410 ("the Sponsor")

From: St George's Hospital NHS Trust, Blackshaw Road Tooting, London SW17 0QT and St George's Hospital Medical School, Cranmer Terrace, Tooting, London SW17 0RE ("the Authorities")

Re: Clinical Study No: C02-001, "An Open-Label Pilot Study of the Effect of Eculizumab (h5G1.1-mAb) on Patients with Transfusion Dependent, Hemolytic, Paroxymal Nocturnal Hemoglobinuria"

1. It is proposed that the Authorities should agree to participate in the above sponsored study ("the Study") involving patients of the Authorities ("the Subjects") to be conducted by ("the Investigator") in accordance with the protocol annexed, as amended from time to time with the agreement of the Sponsor and the Investigator ("the Protocol"). The Sponsor confirms that it is a term of its agreement with the Investigator that the Investigator shall obtain all the necessary approval of the applicable Local Research Ethics Committee and shall resolve with the Authorities any issues of a revenue nature.

2. The Authorities agrees to participate by allowing the Study to be undertaken on its premises utilising such facilities, personnel and equipment as the Investigator may reasonably need for the purpose of the Study.

3. In consideration of such participation by the Authorities, and subject to paragraph 4 below, the Sponsor indemnifies and holds harmless the Authorities and its employees and agents against all claims and proceedings (to include any settlements or ex-gratis payments made with the consent of the parties hereto and reasonable legal and expert costs and expenses) made or brought (whether successfully or otherwise)

   a) by or on behalf of Subjects taking part in the study (or their dependants) against the Authorities or any of its employees or agents for personal injury (including death) to Subjects arising out of or relating to the administration of the product(s) under investigation or any clinical intervention or procedure provided for or required by the Protocol to which the Subjects would not have been exposed but for their participation in the Study.

   b) by or on behalf of a Subject for a declaration concerning the treatment of a Subject who has suffered such personal injury.

4. The above indemnity by the Sponsor shall not apply to any such claim or proceeding:

   4.1 to the extent that such personal injury (including death) is caused by the negligent or wrongful acts or omissions or breach of statutory duty of the Authorities, its employees or agents.

   4.2 to the extent that such personal injury (including death) is caused by the failure of the Authorities, its employees, or agents to conduct the Study in accordance with the Protocol.

4.3 unless as soon as reasonably practicable following receipt of notice of such claim or proceeding, the Authorities shall have notified the Sponsor in writing of it and shall, upon the Sponsor's request, and at the Sponsor's cost, have permitted the Sponsor to have full care and control of the claim or proceeding using legal representation of its own choosing.

4.4 If the Authorities, its employees, or agents shall have made any admission in respect of such claim or proceeding or taken any action relating to such claim or proceeding prejudicial to the defence of it without the written consent of the Sponsor such consent not to be unreasonably withheld provided that this condition shall not be treated as breached by any statement properly made by the Authorities, its employees or agents in connection with the operation of the Authorities internal complaint procedures, accident reporting procedures or disciplinary procedures or where such statement is required by law.

5. The Sponsor shall keep the Authorities and its legal advisers fully informed of the progress of any such claim or proceeding, will consult fully with the Authorities on the nature of any defence to be advanced and will not settle any such claim or proceeding without written notice to the Authorities.

6. Without prejudice to the provisions of paragraph 4.3 above, the Authorities will use its reasonable endeavours to inform the Sponsor promptly of any circumstances reasonably thought likely to give rise to any such claim or proceeding of which it is directly aware and shall keep the Sponsor reasonably informed of developments in relation to any such claim or proceeding even where the Authorities decides not to make a claim under this indemnity. Likewise, the Sponsor shall use its reasonable endeavours to inform the Authorities of any such circumstances directly involving the Subjects and shall keep the Authorities reasonably informed of developments in relation to any such claim or proceeding made or brought against the Sponsor alone.

7. The Authorities and the Sponsor will each give to the other such help as may reasonably be required for the efficient conduct and prompt handling of any claim or proceeding by or on behalf of Subjects (or their dependants) or concerning such a declaration as is referred to in paragraph 3(b) above.

8. Without prejudice to the foregoing if injury is suffered by a Subject while participating in the Study, the Sponsor agrees to operate in good faith the Guidelines published in 1991 by The Association of the British Pharmaceutical Industry and entitled "Clinical Trial Compensation Guidelines" (where the Subject is a patient) and the Guidelines published in 1988 by the same Association and entitled "Guidelines for Medical Experiments in non-patient Human Volunteers" (where the Subject is not a patient) and shall request the Investigator to make clear to the Subjects that the Study is being conducted subject to the applicable Association Guidelines.

9. For the purpose of this indemnity, the expression "agents" shall be deemed to include without limitation any nurse or other health professional providing services to the Authorities under a contract for services or otherwise and any person carrying out work for the Authorities under such a contract connected with such of the Authorities facilities and equipment as are made available for the Study under paragraph 2 above.

10. This indemnity shall be governed by and construed in accordance with English law.

SIGNED on behalf of the Health Authority

.....
Chief Executive/District General Manager

.....
Dated

SIGNED on behalf of the Sponsor

.....
Thomas I. H. Dubin
Vice President and General Counsel

Dated.....

IN THE MATTER OF
European Patent No. EP 2 359 834

DECLARATION OF DR. DINO MIANO

I, Dr. Dino Miano, hereby declare as follows:

1. I am Dr. Dino Miano of Alexion Pharmaceuticals, Inc., 100 College St, New Haven, Connecticut, USA. I hold a Ph.D. degree in Chemistry and Biochemistry from the University of Massachusetts, and a Bachelor of Science degree in Chemistry and Biology from the University of Hartford. I have been employed by Alexion Pharmaceuticals for nearly 20 years, and I currently hold the post of Executive Director, Global Analytical & Pharmaceutical Development. Prior to this I was employed by R.W. Johnson Pharmaceutical Research Institute in Raritan, New Jersey for approximately two years. During my employment at Alexion my responsibilities and work have focused on analytical sciences and protein characterization. I have significant expertise in formulation development and analysis, which includes designing, performing and overseeing experiments that assess the impact of protein concentration, formulation, and storage conditions on the integrity and stability of a protein over time. A copy of my resume is attached as Exhibit 1.

2. The commercial formulation of Soliris®(eculizumab) consists of a concentration of 10 mg/mL of eculizumab in a buffer of 10 mM sodium phosphate, 150 mM sodium chloride and 0.02% polysorbate 80 at pH 7.0. The commercial formulation is satisfactorily stable for 18 months when it is stored as drug substance (i.e., in bulk in the commercial formulation) and for an additional 30 months when it is stored as a drug product (i.e., in 30 mL vials as it is supplied to consumers) providing a total of 48 months of stability. (See March 16, 2007 BLA Approval Letter from U.S. FDA, attached as Exhibit 2 (indicating the drug substance is stable for 18 months and the drug product is stable for 24 months at 2-8°C), and May 8, 2009 Letter from U.S. FDA, attached as Exhibit 3 (extending the shelf-life of the drug product from 24 to 30 months at 2-8°C after supplemental stability data was obtained).)

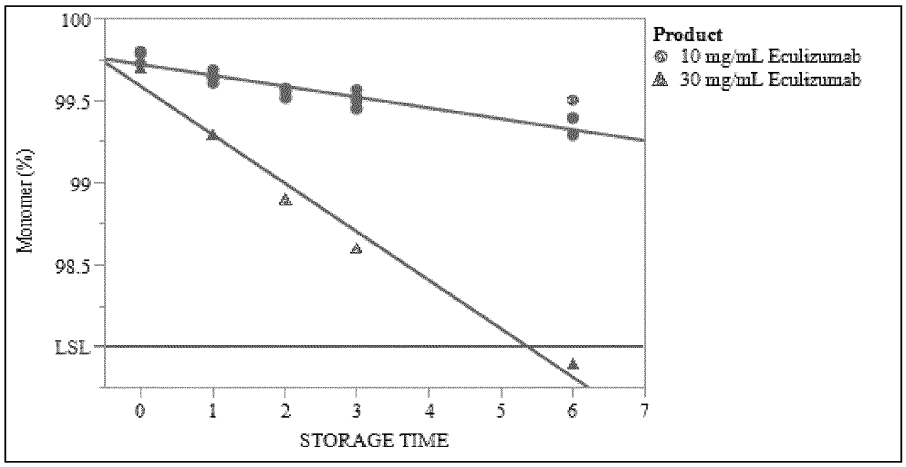
3. One of the stability parameters that the commercial formulation of eculizumab must meet in order to be deemed satisfactorily stable and thus suitable for release into the market is the percent of monomer antibody in the sample must be greater than or equal to 98.0%, which means that there must be less than or equal to 2.0% aggregates of the antibody, as determined via gel permeation high performance liquid chromatography. This release parameter for commercial lots of eculizumab ensures that the drug is satisfactorily stable for the designated storage period.

4. We generated a 30 mg/mL formulation of eculizumab (internally denoted "ALXN5100") that was identical to the commercial formulation of eculizumab, except that ALXN5100 has an eculizumab concentration of 30 mg/mL and the Soliris® commercial formulation has an eculizumab concentration of 10 mg/mL.

5. A batch of ALXN5100 was made under Good Manufacturing Practices (GMP) conditions, and stability studies on the batch were conducted by aliquotting samples into representative HyQ® CX5-14 Hyclone bags to simulate large-scale storage. The Hyclone bags were then stored horizontally at 2 to 8°C for at least 18 months and at 23 to 27°C for at least 6 months, which is how samples were stored for the stability program for the bulk drug substance for the commercial (10 mg/mL) formulation. Although the commercial formulation of eculizumab is to be stored at 2 to 8°C, when performing stability testing, researchers often store test samples at higher temperatures than commercial supplies are typically stored at, such as 23 to 27°C, because storage and stability testing at higher temperatures can provide a good indication of the stability of the formulation in a shorter test period of time. Gel Permeation High Performance Liquid Chromatography (GP-HPLC) was used to determine if there were any changes in monomer and aggregate drug at the two storage temperatures over time.

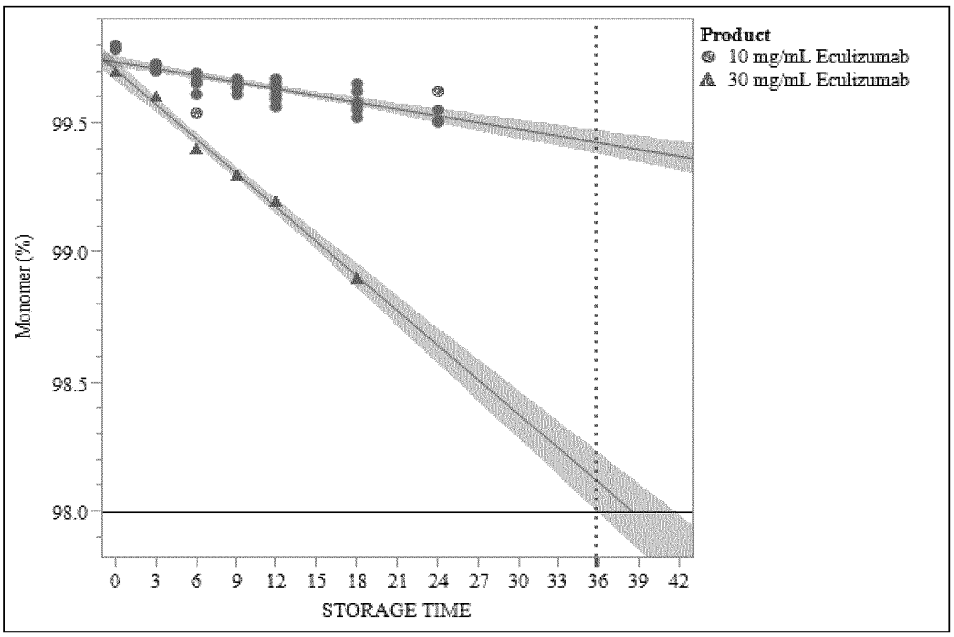
6. Figures 1 and 2 below demonstrate that the stability of eculizumab at 10 mg/mL is superior to that at 30 mg/mL. Figure 1 compares the percent monomer in the 10 mg/mL eculizumab composition versus the percent monomer in the 30 mg/mL eculizumab composition after storage at 23 to 27°C over six months. Figure 1 clearly shows that the percent monomer of eculizumab decreases (and thus aggregate formation increases) significantly more quickly in the 30 mg/mL eculizumab composition than in the 10 mg/mL eculizumab composition. In addition, whereas the 10 mg/mL eculizumab composition remains sufficiently stable over the specification ("LSL") monomer level of 98.0% over the entire six-month period, the 30 mg/mL eculizumab composition falls below that stability threshold during the same time period.

Figure 1. Percent monomer of eculizumab in 10 mg/mL eculizumab composition versus 30 mg/mL eculizumab composition over six months when stored at 23-27°C



7. Similar trends are seen between the 10 mg/mL eculizumab composition and the 30 mg/mL eculizumab composition even at storage temperatures of 2 to 8°C. Figure 2 compares the percent monomer in the 10 mg/mL eculizumab composition versus the percent monomer in the 30 mg/mL eculizumab composition after storage at 2 to 8°C over 18 months. Again, as with the 23 to 27°C storage conditions, the percent monomer remaining in the 30 mg/mL eculizumab samples decreased much more rapidly than the percent monomer remaining in the 10 mg/mL eculizumab samples at 2 to 8°C over the 18-month period. In addition, the model generated from this data predicted that the 30 mg/mL eculizumab composition would fall below the acceptable 98.0% monomer level before 36 months of storage.

Figure 2. Percent monomer of eculizumab in 10 mg/mL eculizumab composition versus 30 mg/mL eculizumab composition over 18 months when stored at 2-8°C



8. Thus, these long-term stability studies demonstrate a decrease in monomer and an increase in aggregate formation for ALXN5100, the 30 mg/mL eculizumab concentration, throughout the stability program. In all instances the aggregate formation in the 30 mg/mL concentration was greater than what was observed in the 10 mg/mL concentration. These results are clearly demonstrated through 18 months at 2 to 8°C and are even more pronounced within 6 months at the higher temperature studies. These results suggest that the commercial formulation is optimal for an eculizumab concentration of 10 mg/mL but is not suitable when the eculizumab concentration is increased to 30 mg/mL.

I believe and do hereby declare that the facts stated above are to the best of my knowledge true.

Dr. Dino Miano

17 Mar 2018
Date

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Exhibit 1

Dino C. Miano, Ph. D.
217 Sunset Ridge
Rocky Hill, CT 06067
(860) 563-5433 (Home)
(860) 983-6178 (Mobile)
dinomiano@sbcglobal.net

QUALIFICATION PROFILE

- Results-oriented professional with eighteen years' successful track record of innovation, management, in depth scientific and regulatory knowledge in analytical and pharmaceutical development in the field of biotherapeutics. Experience encompasses various stages of biologics including early and late phase development, regulatory submission for marketing authorization and life cycle management.

- Well established division-level leader that consistently drives vision toward successful and timely completion of projects, while focusing on translating goals into actionable strategies that support the business

COMPETENCIES

- Pharmaceutical (biological) development
  - Formulation development
  - Fill finish
  - Stability monitoring and trending
  - Device development
  - Analytical support of clinical operations
- Regulatory Compliance
  - CMC development
- Analytical Development
  - Method Validation & Transfer
  - GMP Testing & Compliance
  - Implementing new analytical technologies
  - Analytical support of manufacturing
- Strategic leadership and innovation
  - Cross functional leader

Accomplishments and Qualifications

- Assumed a key analytical role in the clinical development and global commercialization of Soliris® and Strensiq®. Led development and validation activities for commercial release and stability test methods.
- Lead Global Operations efforts in delivering clinical material from early stage through commercial. Lead cross functional teams consisting of manufacturing, supply chain, QA and CMC and working with clinical groups to ensure supply demands are consistently met
- Lead Global Analytical and Pharmaceutical Development groups in providing analytical support to the entire Alexion organization, including analytical development, validation and transfer, GMP testing of clinical products, process development support, formulation and device development, product characterization and stability to allow for expiry determination.
- Implemented GMP quality infrastructure in the Analytical Sciences laboratories to ensure testing compliance with FDA, EU and PMDA regulations.
- Lead formulation development of new, existing and commercial products for IV, IVT and subcutaneous administration.

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- Implement drug substance and drug product specifications for clinical products
- Generate analytical sections in support of all new and existing CMC submissions
- Lead the drug delivery and device development group in developing a strategy and business process to advance drug and device/combination products for existing commercial and clinical products.

Employment History

Alexion Pharmaceuticals, Inc., New Haven, CT

Executive Director, Global Analytical and Pharmaceutical Development **12/15-present**

- Lead global analytical support function for the entire organization for all commercial and clinical programs.
- Lead the global method technology transfers for Alexion's clinical and commercial products.
- Oversee compatibility assessments for materials used for delivery of product to patients during clinical studies.
- Represent Global Operations as lead for late stage process development, drug substance and drug product manufacturing, supply chain and CMC/IND submissions.
- Lead the device group in collaboration with internal and external partners for the development of delivery devices for Alexion products.

Senior Director, Analytical and Pharmaceutical Development **03/15-12/15**

- Led Early Stage Analytical and Product Characterization groups in supporting Research in efforts to move the best candidates into development.
- Led Analytical Sciences in method development, validation and transfer for new candidates and provide support to process development groups.
- Lead the stability program for all clinical products, including expiry determinations
- Led and aligned formulation development of new candidates with Clinical development and Target product profiles (TPP).
- Led Device Development and Drug Delivery group in identifying and integrating large volume bolus injector options for clinical and commercial development.
- Appointed CMC lead during Regulatory meetings with FDA, Health Canada, EMA and MFDS (F2F) to support CMC section of regulatory filings.

Director, Product Characterization **07/14-03/15**

- Led Global Operations Cross Functional Product Team in supporting second generation development of commercial product.
- Led device teams consisting of Clinical and Commercial Operations, Development, Regulatory and QA in evaluating large volume subcutaneous administration devices. Device included auto-injectors and larger wearables patch devices.
- Managed Mass Spectroscopy Characterization group. Involves the MS characterization of new and existing molecules to support CMC, Research and Development groups.
- Managed the Bio-Physical Characterization group. Involves directing the testing and collaboration of multiple groups to better understand new protein therapeutics
- Provided analytical support of new and existing CMC submissions and address inquiries from agencies

Director, Analytical Sciences **09/08-07/14**

- Managed the Operations group that provided GMP testing of clinical products and analytical testing to all development groups.
- Managed the Analytical Development group that provided method development, qualification, validation and transfer of analytical methods for new clinical products and existing commercial product to both QC and EU laboratories.
- Manage the Formulation Development group that identified stable formulations for new clinical products and determined stable subcutaneous formulations for both clinical and commercials products.
- Managed Stability program of clinical products that generated GMP stability protocols and reports, trended data and provided statistical analysis of trends to support expiry, and led deviations, LIR and OOS for GMP testing.
- Member of Specification committee to direct the generation or revision of specifications for clinical products

Associate Director, Analytical Sciences **05/03-08/08**

- Analytical Group
  - Provided analytical support of new and existing Soliris® CMC submissions
  - Supervised Analytical Operations laboratory in providing method development, qualification, validation, transfer and GMP testing to support clinical products.
- Purification Development group:
  - Managed PD studies in support of Process Validation. Studies included viral clearance, DNA clearance, process limits evaluation (PLE), resin re-use, and UF/DF membrane re-use.

Manager, Quality Control **08/99-05/03**

- Managed QC laboratories; maintained cGMP compliance with regards to current SOPs, validation/qualifications, and training.
- Coordinated studies, supervised investigations, generated reports and implemented systems with QA in response to OOS, invalid assays, aberrant trends/results.
- Represented area functions as required during audits by external agencies and internal QA department.

Senior Scientist, Quality Control **08/98-08/99**

- Developed and optimized RP-HPLC, SEC-HPLC, SDS-PAGE, and IEF methods for all protein based therapeutic product.

R.W. Johnson Pharmaceutical Research Institute, Raritan, NJ
A Johnson & Johnson Company

Scientist, Analytical Development **07/97-07/98**

- Developed HPLC assays for quantitation of becaplermin drug substance in alternative REGRANEX® formulations. Developed HPLC assays for determining content of preservatives in REGRANEX® formulations.

Scientist, Protein Development **01/97-07/97**

- Investigated modified roller bottle processes for the production of erythropoietin (rHuEPO), potentially increasing production/yields by 3-fold.
- Evaluated alternative purification processes for rHuEPO, involving HIC, IEC and Reverse phase chromatography.

Postdoctoral Research Scientist, Protein Development **10/95-01/97**

- Chemically modified rHuEPO using polyethylene glycol (PEG) to enhance in vivo erythropoiesis while prolonging circulatory half-life. Experiments were designed to specifically modify the carbohydrate (NANA) moieties of the glycoprotein with PEG while maintaining the native polypeptide conformation. Animal model analysis revealed successful prolonged in vivo biological activity.

University of Connecticut Health Center, Farmington, CT **01/89-08/90**

Research Scientist, Surgery Department

- Investigated/detected tissue derived inflammatory mediators released from injured corneas, urinary bladders and ischemic hearts. Modified Boyden chambers were employed to evaluate chemotactic response via neutrophil migration through 8 μm membranes.
- Investigated/synthesized cyclocreatine, a creatine analog, to determine inhibitory effects on the production and release of neutrophil chemotactic factors (NCF) in ischemic canines and rabbits myocardium.

EDUCATION

University of Massachusetts, Lowell, MA **09/90-10/95**

Ph. D. Chemistry/Biochemistry
Advisor: Dr. Edwin G.E. Jahngen
Thesis: "The Isolation and characterization of an ubiquitin-histone protein from *Dictyostelium discoideum AX3.*"

University of Hartford, Bloomfield, CT **09/85-05/89**

B.S. Chemistry/Biology
Research: "Corneal derived neutrophil chemotactic factors, intracellular synthesis and release."

PUBLICATIONS

Neill, A., Nowak, C., Patel, R., Ponniah, G., Gonzalez, N., **Miano, D**. and Liu, H., (2015) Characterization of recombinant monoclonal antibody charge variants using OFFGEL fractionator, weak anion exchange chromatography and mass spectrometry, *Analytical Chemistry*, **87**, 6204-6211

Ponniah, G., Nowak, C., Gonzalez, N., **Miano D**., and Liu, H., (2015) Detection and quantitation of low abundance oligosaccharides in recombinant monoclonal antibodies. *Analytical Chemistry*, **87**, 2718-2726

Peckham, N., Neil, F., and **Miano, D.C**., "Quantitative SDS-PAGE Analysis of a Recombinant Protein by Densitometry", IBC's Well Characterized Biologicals Poster Session, Washington D.C. (11/11/04)

Peckham, N., Bosley, K., and **Miano, D.C**., Adaptation of Complexometric Titration Method for Quantitation of EDTA in Biopharmaceutical Formulations to Microplate Format, IBC's Well Characterized Biologicals Poster Session, Washington D.C. (11/11/04).

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Exhibit 2

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service
Food and Drug Administration
Rockville, MD 20857

Our STN: BL 125166/0

MAR 16 2007

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

Dear Dr. Motola:

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

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

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

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

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

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

You must submit information to your biologics license application for our review and written approval under 21 CFR 601.12 for any changes in the manufacturing, testing, packaging or labeling of eculizumab, or in the manufacturing facilities.

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

Postmarketing Studies subject to reporting requirements of 21 CFR 601.70.

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

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

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

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

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

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

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

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

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

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

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

      i. Death;

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

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

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

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

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

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

ii. Soliris use patterns including indication for use;

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

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

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

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

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

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

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

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

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

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

8. To revalidate the linearity of the IEF method across a load range of 8 µg to 12 µg. The revalidation plan will be submitted in advance to FDA for review and endorsement. This information will be submitted to the BLA as a CBE 30 supplement by August 31, 2007.

9. To revise the IEF method SOP to specify that the method is validated only for a 10 µg load. This information will be submitted to the BLA as a CBE 30 by August 31, 2007.

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

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

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

13. To develop a suitable antifoam assay and subsequently confirm the removal of antifoam from the process stream at the Protein A chromatography pool on three drug substance batches. This information will be submitted to the BLA as a CBE 30 by August 31, 2007.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Sincerely,

Karen Weiss for

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

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

Soliris ™ (eculizumab)

FULL PRESCRIBING INFORMATION

WARNING: SERIOUS MENINGOCOCCAL INFECTION

Soliris increases the risk of meningococcal infections (5.1)

- **Vaccinate patients with a meningococcal vaccine at least 2 weeks prior to receiving the first dose of Soliris; revaccinate according to current medical guidelines for vaccine use**
- **Monitor patients for early signs of meningococcal infections, evaluate immediately if infection is suspected, and treat with antibiotics if necessary.**

1 INDICATIONS AND USAGE

Soliris is indicated for the treatment of patients with paroxysmal nocturnal hemoglobinuria (PNH) to reduce hemolysis.

2 DOSAGE AND ADMINISTRATION

Patients must be administered a meningococcal vaccine at least two weeks prior to initiation of Soliris therapy and revaccinated according to current medical guidelines for vaccine use. [*see Warnings and Precautions (5.1)*].

2.1 Recommended Dosage Regimen

Soliris therapy consists of

- 600 mg every 7 days for the first 4 weeks, followed by
- 900 mg for the fifth dose 7 days later, then
- 900 mg every 14 days thereafter.

Soliris should be administered at the recommended dosage regimen time points, or within two days of these time points. [*see Warnings and Precautions (5.5)*]

2.2 Preparation for Administration

Soliris must be diluted to a final admixture concentration of 5 mg/mL using the following steps:

- Withdraw the required amount of Soliris from the vial into a sterile syringe.
- Transfer the recommended dose to an infusion bag.
- Dilute Soliris to a final concentration of 5 mg/mL by adding the appropriate amount (equal volume of diluent to drug volume) of 0.9% Sodium Chloride Injection, USP; 0.45% Sodium Chloride Injection, USP; 5% Dextrose in Water Injection, USP; or Ringer's Injection, USP to the infusion bag.

The final admixed Soliris 5 mg/mL infusion volume is 120 mL for 600 mg doses or 180 mL for 900 mg doses. Gently invert the infusion bag containing the diluted Soliris solution to ensure thorough mixing of the product and diluent. Discard any unused portion left in a vial, as the product contains no preservatives.

Prior to administration, the admixture should be allowed to adjust to room temperature [18°-25° C, 64-77° F]. The admixture must not be heated in a microwave or with any heat source other than ambient air temperature. The Soliris admixture should be inspected visually for particulate matter and discoloration prior to administration.

2.3 Administration

Do Not Administer As An Intravenous Push Or Bolus Injection

The Soliris admixture should be administered by intravenous infusion over 35 minutes via gravity feed, a syringe-type pump, or an infusion pump. Admixed solutions of Soliris are stable for 24 hours at 2-8° C (36-46° F) and at room temperature.

If an adverse reaction occurs during the administration of Soliris, the infusion may be slowed or stopped at the discretion of the physician. If the infusion is slowed, the total infusion time should not exceed two hours. Monitor the patient for at least one hour following completion of the infusion for signs or symptoms of an infusion reaction.

3 DOSAGE FORMS AND STRENGTHS

Soliris is supplied as 300 mg single-use vials each containing 30 mL of 10 mg/mL sterile, preservative-free eculizumab solution.

4 CONTRAINDICATIONS

Do not initiate Soliris therapy in patients:

- with unresolved serious *Neisseria meningitidis* infection.
- who are not currently vaccinated against *Neisseria meningitidis*.

5 WARNINGS AND PRECAUTIONS

5.1 Serious Meningococcal Infections

The use of Soliris increases a patient's susceptibility to serious meningococcal infections (septicemia and/or meningitis). All patients without a history of prior meningococcal vaccination must receive the meningococcal vaccine at least 2 weeks prior to receiving the first dose of Soliris and revaccinated according to current medical guidelines for vaccine use. Quadravalent, conjugated meningococcal vaccines are strongly recommended. Vaccination may not prevent meningococcal infections.

All patients must be monitored for early signs and symptoms of meningococcal infections and evaluated immediately if an infection is suspected. Physicians should strongly consider discontinuation of Soliris during the treatment of serious meningococcal infections.

In clinical studies, 2 out of 196 PNH patients developed serious meningococcal infections while receiving treatment with Soliris; both had been vaccinated. [*see Adverse Reactions (6.1)*].

5.2 Other Infections

Soliris blocks terminal complement; therefore patients may have increased susceptibility to infections, especially with encapsulated bacteria. Use caution when administering Soliris to patients with any systemic infection.

5.3 Monitoring After Soliris Discontinuation

Since Soliris therapy increases the number of PNH cells [in study 1, the proportion of PNH RBCs increased among Soliris-treated patients by a median of 28% from baseline (range from -25% to 69%)], patients who discontinue treatment with Soliris may be at increased risk for serious hemolysis. Serious hemolysis is identified by serum LDH levels greater than the pre-treatment level, along with any of the following: greater than 25% absolute decrease in PNH clone size (in the absence of dilution due to transfusion) in one week or less; a hemoglobin level of <5 gm/dL or a decrease of >4 gm/dL in one week or less; angina; change in mental status; a 50% increase in serum creatinine level; or thrombosis. Monitor any patient who discontinues Soliris for at least 8 weeks to detect serious hemolysis and other reactions.

If serious hemolysis occurs after Soliris discontinuation, consider the following procedures/treatments: blood transfusion (packed RBCs), or exchange transfusion if the PNH RBCs are >50% of the total RBCs by flow cytometry; anticoagulation; corticosteroids; or reinstitution of Soliris.

In clinical studies, 16 of 196 PNH patients discontinued treatment with Soliris. Patients were followed for evidence of worsening hemolysis and no serious hemolysis was observed.

5.4 Thrombosis Prevention and Management

The effect of withdrawal of anticoagulant therapy during Soliris treatment has not been established. Therefore, treatment with Soliris should not alter anticoagulant management.

5.5 Laboratory Monitoring

Serum LDH levels increase during hemolysis and may assist in monitoring Soliris effects, including the response to discontinuation of therapy. In clinical studies, six patients achieved a reduction in serum LDH levels only after a decrease in the Soliris dosing interval from 14 to 12 days. All other patients achieved a reduction in serum LDH levels with the 14 day dosing interval [*see Clinical Pharmacology (12.2) and Clinical Studies (14)*].

5.6 Infusion Reactions

As with all protein products, administration of Soliris may result in infusion reactions, including anaphylaxis or other hypersensitivity reactions. In clinical trials, no PNH patients experienced an infusion reaction which required discontinuation of Soliris. Soliris administration should be interrupted in all patients experiencing severe infusion reactions and appropriate medical therapy administered.

6 ADVERSE REACTIONS

6.1 Clinical Trial Experience

Meningococcal infections are the most important adverse reactions experienced by patients receiving Soliris therapy. In PNH clinical studies, two patients experienced meningococcal sepsis. Both patients had previously received a meningococcal vaccine. In clinical studies among patients without PNH, meningococcal meningitis occurred in an unvaccinated patient [*see Warnings and Precautions (5.1)*].

The data described below reflect exposure to Soliris in 196 adult patients with PNH, age 18-85, of whom 55% were female. All had signs or symptoms of intravascular hemolysis. Soliris was studied in a placebo-controlled clinical study (in which 43 patients received Soliris and 44, placebo); a single arm clinical study and a long term extension study. 182 patients were exposed for greater than one year. All patients received the recommended Soliris dose regimen.

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice. Table 1 summarizes the adverse reactions that occurred at a numerically

higher rate in the Soliris group than the placebo group and at a rate of 5% or more among patients treated with Soliris.

TABLE 1

ADVERSE REACTIONS REPORTED IN 5% OR MORE OF SOLIRIS TREATED PATIENTS AND GREATER THAN PLACEBO IN THE CONTROLLED CLINICAL STUDY

Reaction	Soliris N = 43 N (%)	Placebo N = 44 N (%)
Headache	19 (44)	12 (27)
Nasopharyngitis	10 (23)	8 (18)
Back pain	8 (19)	4 (9)
Nausea	7 (16)	5 (11)
Fatigue	5 (12)	1 (2)
Cough	5 (12)	4 (9)
Herpes simplex infections	3 (7)	0
Sinusitis	3 (7)	0
Respiratory tract infection	3 (7)	1 (2)
Constipation	3 (7)	2 (5)
Myalgia	3 (7)	1 (2)
Pain in extremity	3 (7)	1 (2)
Influenza-like illness	2 (5)	1 (2)

In the placebo-controlled clinical study, serious adverse reactions occurred among 4 (9%) patients receiving Soliris and 9 (21%) patients receiving placebo. The serious reactions included infections and progression of PNH. No deaths occurred in the study and no patients receiving Soliris experienced a thrombotic event; one thrombotic event occurred in a patient receiving placebo.

Among 193 patients with PNH treated with Soliris in the single arm, clinical study or the follow-up study, the adverse reactions were similar to those reported in the placebo-controlled clinical study. Serious adverse reactions occurred among 16% of the patients in these studies. The most common serious adverse reactions were: viral infection (2%), headache (2%), anemia (2%), and pyrexia (2%).

6.2 Immunogenicity

As with all proteins there is a potential for immunogenicity. Low titers of antibodies to Soliris were detected in 3/196 (2%) of all PNH patients treated with Soliris. No apparent correlation of antibody development to clinical response was observed. The immunogenicity data reflect the percentage of patients whose test results were considered positive for antibodies to Soliris in an enzyme linked immunosorbent assay (ELISA) and are highly dependent on the sensitivity and specificity of the assay. Additionally, the observed incidence of antibody positivity in the assay may be influenced by several factors including sample handling, timing of sample collection, concomitant medications and underlying disease. For these reasons, comparison of the incidence of antibodies to Soliris with the incidence of antibodies to other products may be misleading.

7 DRUG INTERACTIONS

Drug interaction studies have not been performed with Soliris.

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Pregnancy Category C:

PNH is a serious illness. Pregnant women with PNH and their fetuses have high rates of morbidity and mortality during pregnancy and the postpartum period. There are no adequate and well-controlled studies of Soliris in pregnant women. Soliris, a recombinant IgG molecule (humanized anti-C5 antibody), is expected to cross the placenta. Animal studies using a mouse analogue of the Soliris molecule (murine anti-C5 antibody) showed increased rates of developmental abnormalities and an increased rate of dead and moribund offspring at doses 2-8 times the human dose. Soliris should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Animal reproduction studies were conducted in mice using doses of a murine anti-C5 antibody that approximated 2-4 times (low dose) and 4-8 times (high dose) the recommended human Soliris dose, based on a body weight comparison. When animal exposure to the antibody occurred in the time period from before mating until early gestation, no decrease in fertility or reproductive performance was observed. When maternal exposure to the antibody occurred during organogenesis, two cases of retinal dysplasia and one case of umbilical hernia were observed among 230 offspring born to mothers exposed to the higher antibody dose; however, the exposure did not increase fetal loss or neonatal death. When maternal exposure to the antibody occurred in the time period from implantation through weaning, a higher number of male offspring became moribund or died (1/25 controls, 2/25 low dose group, 5/25 high dose group). Surviving offspring had normal development and reproductive performance.

8.2 Labor and Delivery

No information is available on the effects of Soliris during labor and delivery.

8.3 Nursing Mothers

It is not known whether Soliris is secreted into human milk. IgG is excreted in human milk, so it is expected that Soliris will be present in human milk. However, published data suggest that breast milk antibodies do not enter the neonatal and infant circulation in substantial amounts. Caution should be exercised when Soliris is administered to a nursing woman. The unknown risks to the infant from gastrointestinal or limited systemic exposure to Soliris should be weighed against the known benefits of breastfeeding.

8.4 Pediatric Use

The safety and effectiveness of Soliris therapy in pediatric patients below the age of 18 have not been established.

8.5 Geriatric Use

In PNH studies, 15 patients 65 years of age or older were treated with Soliris. Although there were no apparent age-related differences observed in these studies, the number of patients aged 65 and over is not sufficient to determine whether they respond differently from younger patients.

10 OVERDOSAGE

No cases of Soliris overdose have been reported during clinical studies.

11 DESCRIPTION

Soliris is a formulation of eculizumab which is a recombinant humanized monoclonal $IgG_{2/4}\kappa$ antibody produced by murine myeloma cell culture and purified by standard bioprocess technology. Eculizumab contains human constant regions from human IgG2 sequences and human IgG4 sequences and murine complementarity-determining regions grafted onto the human framework light- and heavy-chain variable regions. Eculizumab is composed of two 448 amino acid heavy chains and two 214 amino acid light chains and has a molecular weight of approximately 148 kDa.

Soliris is a sterile, clear, colorless, preservative-free 10 mg/mL solution for intravenous infusion and is supplied in 30-mL single-use vials. The product is formulated at pH 7 and each vial contains 300 mg of eculizumab, 13.8 mg sodium phosphate monobasic, 53.4 mg sodium phosphate dibasic, 263.1 mg sodium chloride, 6.6 mg polysorbate 80 (vegetable origin) and Water for Injection, USP.

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

Eculizumab, the active ingredient in Soliris, is a monoclonal antibody that specifically binds to the complement protein C5 with high affinity, thereby inhibiting its cleavage to C5a and C5b and preventing the generation of the terminal complement complex C5b-9. Soliris inhibits terminal complement mediated intravascular hemolysis in PNH patients

A genetic mutation in PNH patients leads to the generation of populations of abnormal RBCs (known as PNH cells) that are deficient in terminal complement inhibitors, rendering PNH RBCs sensitive to persistent terminal complement-mediated destruction. The destruction and loss of these PNH cells (intravascular hemolysis) results in low RBC counts (anemia), and also fatigue, difficulty in functioning, pain, dark urine, shortness of breath, and blood clots.

12.2 Pharmacodynamics

In the placebo-controlled clinical study, Soliris when administered as recommended reduced hemolysis as shown by the reduction of serum LDH levels from 2200 ± 1034 U/L (mean ± SD) at baseline to 700 ± 388 U/L by week one and maintained the effect through the end of the study at week 26 (327 ± 433 U/L). In the single arm clinical study, Soliris maintained this effect through 52 weeks [*see Clinical Studies (14)*].

12.3 Pharmacokinetics

A population PK analysis with a standard 1-compartmental model was conducted on the multiple dose PK data from 40 PNH patients receiving the recommended Soliris regimen [*see Dosage and Administration (2.1)*]. In this model, the clearance of Soliris for a typical PNH patient weighing 70 kg was 22 mL/hr and the volume of distribution was 7.7 L. The half-life was 272 ± 82 hrs (mean ± SD). The mean observed peak and trough serum concentrations of Soliris by week 26 were 194 ± 76 mcg/mL and 97 ± 60 mcg/mL, respectively.

Studies have not been conducted to evaluate the PK of Soliris in special patient populations identified by gender, race, age (pediatric or geriatric), or the presence of renal or hepatic impairment.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

Long-term animal studies have not been conducted to evaluate the carcinogenic and genotoxic potential of Soliris. Effects of Soliris upon fertility have not been studied in animals. Intravenous injections of male and female mice with a murine anti-C5 antibody at up to 4-8 times the equivalent of the clinical dose of Soliris had no adverse effects on mating or fertility.

14 CLINICAL STUDIES

The safety and efficacy of Soliris in PNH patients with hemolysis were assessed in a randomized, double-blind, placebo-controlled 26 week study (Study 1); PNH patients were also treated with Soliris in a single arm 52 week study (Study 2); and in a long term extension study. Patients received meningococcal vaccination prior to receipt of Soliris. In all studies, the dose of Soliris was 600 mg study drug every 7 ± 2 days for 4 weeks, followed by 900 mg 7 ± 2 days later, then 900 mg every 14 ± 2 days for the study duration. Soliris was administered as an intravenous infusion over 25 - 45 minutes.

Study 1:

PNH patients with at least four transfusions in the prior 12 months, flow cytometric confirmation of at least 10% PNH cells and platelet counts of at least 100,000/microliter were randomized to either Soliris (n = 43) or placebo (n = 44). Prior to randomization, all patients underwent an initial observation period to confirm the need for RBC transfusion and to identify the hemoglobin concentration (the "set-point") which would define each patient's hemoglobin stabilization and transfusion outcomes. The hemoglobin set-point was less than or equal to 9 g/dL in patients with symptoms and was less than or equal to 7 g/dL in patients without symptoms. Endpoints related to hemolysis included the numbers of patients achieving hemoglobin stabilization, the number of RBC units transfused, fatigue, and health-related quality of life. To achieve a designation of hemoglobin stabilization, a patient had to maintain a hemoglobin concentration above the hemoglobin set-point and avoid any RBC transfusion for the entire 26 week period. Hemolysis was monitored mainly by the measurement of serum LDH levels, and the proportion of PNH RBCs was monitored by flow cytometry. Patients receiving anticoagulants and systemic corticosteroids at baseline continued these medications.

Major baseline characteristics were balanced (see table 2).

TABLE 2
STUDY 1 PATIENT BASELINE CHARACTERISTICS

	Study 1	
Parameter	Placebo N = 44	Soliris N = 43
Mean age (SD)	38 (13)	42 (16)
Gender - female (%)	29 (66)	23 (54)
History of aplastic anemia or myelodysplastic syndrome (%)	12 (27)	8 (19)
Patients with history of thrombosis (events)	8 (11)	9 (16)
Concomitant anticoagulants (%)	20 (46)	24 (56)
Concomitant steroids/immunosuppressant	16 (36)	14 (33)

Parameter	Study 1 Placebo N = 44	 Soliris N = 43
treatments (%)		
Packed RBC units transfused per patient in previous 12 months (median (Q1,Q3))	17 (14, 25)	18 (12, 24)
Mean hgb level (g/dL) at setpoint (SD)	8 (1)	8 (1)
Pre-treatment LDH levels (median, U/L)	2,234	2,032
Free hemoglobin at baseline (median, mg/dL)	46	41

Patients treated with Soliris had significantly reduced ($p < 0.001$) hemolysis resulting in improvements in anemia as indicated by increased hemoglobin stabilization and reduced need for RBC transfusions compared to placebo treated patients (see table 3). These effects were seen among patients within each of the three pre-study RBC transfusion strata (4 - 14 units; 15 - 25 units; > 25 units). After 3 weeks of Soliris treatment, patients reported less fatigue and improved health-related quality of life. Because of the study sample size and duration, the effects of Soliris on thrombotic events could not be determined.

TABLE 3
STUDY 1 RESULTS

	Placebo N = 44	Soliris N = 43
Percentage of patients with stabilized hemoglobin levels	0	49
Packed RBC units transfused per patient (median)	10	0
(range)	(2 - 21)	(0 - 16)
Transfusion avoidance (%)	0	51
LDH levels at end of study (median, U/L)	2,167	239
Free hemoglobin at end of study (median, mg/dL)	62	5

Study 2 and Extension Study:
PNH patients with at least one transfusion in the prior 24 months and at least 30,000 platelets/microliter received Soliris over a 52-week period. Concomitant medications included anti-thrombotic agents in 63% of the patients and systemic corticosteroids in 40% of the patients. Overall, 96 of the 97 enrolled patients completed the study (one patient died following a thrombotic event). A reduction in intravascular hemolysis as measured by serum LDH levels was sustained for the treatment period and resulted in a reduced need for RBC transfusion and less fatigue. 187 Soliris-treated PNH patients were enrolled in a long term extension study. All patients sustained a reduction in intravascular hemolysis over a total Soliris exposure time ranging from 10 to 54 months. There were fewer thrombotic events with Soliris treatment than during the same period of time prior to treatment. However, the majority of patients received concomitant anticoagulants; the effects of anticoagulant withdrawal during Soliris therapy was not studied [*see Warnings and Precautions (5.4)*].

16 HOW SUPPLIED / STORAGE AND HANDLING

Soliris (eculizumab) is supplied as 300 mg single-use vials containing 30 mL of 10 mg/mL sterile, preservative-free Soliris solution per vial.

Soliris vials must be stored in the original carton until time of use under refrigerated conditions at 2-8° C (36-46° F) and protected from light. Do not use beyond the expiration date stamped on the carton. Refer to *Dosage and Administration* (2) for information on the stability and storage of diluted solutions of Soliris.

DO NOT FREEZE. DO NOT SHAKE.

NDC 25682-001-01 Single unit 300 mg carton: Contains one (1) 30 mL vial of Soliris (10 mg/mL).

17 PATIENT COUNSELING INFORMATION

See Medication Guide.

Prior to treatment, patients should fully understand the risks and benefits of Soliris, in particular the risk of meningococcal infection. Ensure that patients receive the Medication Guide.

Patients should be informed that they are required to receive a meningococcal vaccination at least 2 weeks prior to receiving the first dose of Soliris, if they have not previously been vaccinated. They are required to be revaccinated according to current medical guidelines for meningococcal vaccine use while on Soliris therapy. Patients should also be informed that vaccination may not prevent meningococcal infection. Patients should be educated about any of the signs and symptoms of meningococcal infection, and strongly advised to seek immediate medical attention if these signs or symptoms occur. These signs and symptoms are as follows:

- moderate to severe headache with nausea or vomiting
- moderate to severe headache and a fever
- moderate to severe headache with a stiff neck or stiff back
- fever of 103° F (39.4° C) or higher
- fever and a rash
- confusion
- severe muscle aches with flu-like symptoms, and eyes sensitive to light

Patients should be informed that they would be provided with the Patient Safety Card that they should carry with them at all times. This card describes symptoms which, if experienced, should prompt the patient to immediately seek medical evaluation.

Patients should be informed that there is a potential for serious hemolysis when Soliris is discontinued and that they will be monitored by their healthcare professional for at least 8 weeks following Soliris discontinuation.

Manufactured by:

Alexion Pharmaceuticals, Inc.

352 Knotter Drive

Cheshire, CT 06410 USA

US License Number 1743

Patients receiving Soliris treatment should carry this card at all times.

INFORMATION FOR THE PATIENT

You should call your doctor (#_____) right away if you have any of these symptoms:

- **moderate to severe headache with nausea or vomiting**
- **moderate to severe headache and a fever**
- **moderate to severe headache with a stiff neck or stiff back**
- **fever of 103° F (39.4° C) or higher**
- **fever and a rash**
- **confusion**
- **severe muscle aches with flu-like symptoms, and eyes sensitive to light**

If you cannot reach your doctor right away, go to an emergency room and show them this card.

INFORMATION FOR THE PHYSICIAN

This patient was prescribed Soliris (eculizumab) therapy. Soliris is an antibody that inhibits terminal complement activation and prevents the formation of C5b-9 complex. Before beginning treatment, this patient should have received a meningococcal vaccine, but may still be susceptible to meningococcal infections.

For more information about Soliris, see the full prescribing information available at www.soliris.net\soliris.pdf, or call 1-888-SOLIRIS (1-888-765-4747)



Food and Drug Administration
Center for Drug Evaluation and Research
Office of Biotechnology Products

FACSIMILE TRANSMITTAL SHEET

DATE: May 8, 2009

To: Mr. Arthur D. Edwards	**From:** LCDR Andrew Shiber Project Manager
Company: Alexion Pharmaceuticals Corporation	Office of Biotechnology Products
Fax number: 203-271-8191	**Fax number:** (301) 796-9743
Phone number: 203-272-2596	**Phone number:** (301) 796-4798

Subject: STN 125166/36

Total no. of pages including cover: 2

Comments: Please find attached the action letter for the above supplement for Soliris®(eculizumab).

Document to be mailed: X☐ YES ☐ NO

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DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service
Food and Drug Administration
Rockville, MD 20857

STN 125166-36

May 8, 2009

Alexion Pharmaceuticals
Attention: Mr. Arthur D. Edwards
Executive Director, Regulatory Affairs
352 Knotter Drive
Cheshire, CT 06410

Dear Mr. Edwards:

Your request to supplement your biologics license application for Soliris®(eculizumab), to extend the shelf-life of the drug product from 24 to 30 months when stored at 2 – 8 degrees Celsius, has been approved.

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

This information will be included in your biologics license application file.

Sincerely,

Kathleen A. Clouse

Kathleen A. Clouse, Ph.D.
Director
Division of Monoclonal Antibodies
Office of Biotechnology Products
Office of Pharmaceutical Science
Center for Drug Evaluation and Research

Electronic Patent Application Fee Transmittal

Application Number:	15642096
Filing Date:	05-Jul-2017
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/Anita Costa
Attorney Docket Number:	AXJ-114BUSCN5

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

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

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
SUBMISSION- INFORMATION DISCLOSURE STMT	1806	1	240	240
		Total in USD ($)		**240**

Electronic Acknowledgement Receipt	
EFS ID:	37989115
Application Number:	15642096
International Application Number:	
Confirmation Number:	7606
Title of Invention:	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT
First Named Inventor/Applicant Name:	Leonard BELL
Customer Number:	133485
Filer:	Jill R. Gorny Sloper/Anita Costa
Filer Authorized By:	Jill R. Gorny Sloper
Attorney Docket Number:	AXJ-114BUSCN5
Receipt Date:	11-DEC-2019
Filing Date:	05-JUL-2017
Time Stamp:	16:19:21
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$240
RAM confirmation Number	E2019BAG19464127
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Foreign Reference	AXJ-114BUSCN5_-_EP2581113A1.pdf	6886183 fbbe45868ec4868b3359efdefeb0f36ee6a31b91	no	129
Warnings:					
Information:					
2	Foreign Reference	WO2004035607A2.pdf	10212581 d6aa52bd1dc8065c37da8155a32a5acd4cc6a74c	no	201
Warnings:					
Information:					
3	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2045_-_2007_USP_Dictionary_of_USAN_and_Intl_Drug_Names.pdf	1037635 a9bbb815caeca479e3830dc5c7ae84b2998b2ad5	no	85
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Information:					
4	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2060_-_Alexion_10-K___2008_excerpts.pdf	87957 be17789341785c27a29f4bdd9c69abda71635899	no	3
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5	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2061_-_Alexion_10-K___2009_excerpts.pdf	82893 264df0fc65cb6b6e145ed13c248b0340255ed622	no	3
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6	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2063_-_Alexion_10-K___2011_excerpts.pdf	103356 3436ccd708eb7218f2476240654b491eb2897406	no	3
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7	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2059_-_Alexion_10-K___2007_excerpts.pdf	88181 8e63a39bd474e955e7b431a251dd77c05f8b4762	no	3
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8	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2062_-_Alexion_10-K___2010_excerpts.pdf	90291 3918d8e5db10fc746d2e3623a0fc97339c532c11	no	3
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9	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2068_-_Alexion_10-K___2016_excerpts.pdf	82973 3a8194ed8db554d7ab758047f7a7ad3bb58be103	no	3
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11	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2070_-_10-K___2018_02.pdf	105591 2a7d76e9ab26aba0cde09b0c1d9af9b950595b86	no	3
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12	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2071_-_10-Q___Q1_2019.pdf	92778 6797bc39fb719651652caf3fc61ddbd30fe641d7	no	3
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13	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2072_-_10-Q___Q2_2019.pdf	96758 204cf1c4c72075c1bd0bbf6b6c54a05119bdec80	no	3
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14	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2073_-_10-Q___Q3_2019.pdf	98991 625c514008637f895b62b3c2c09f15e392874433	no	3
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16	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2065_-_Alexion_10-K___2013_excerpts.pdf	92686 5f672bb80f3ed0e23d5b2f5d77c96f057ae98f63	no	3
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17	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2066_-_Alexion_10-K___2014_excerpts.pdf	99179 d969a1eb109c3e8a7d618613cd1dcfd45faf6a4a	no	3
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18	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2067_-_Alexion_10-K___2015_excerpts.pdf	101670 3709ac0b56dae8163eef9c8bbc47bffbfd7542cb	no	3
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19	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2070_-_10-K___2018.pdf	105591 4eaa61893ecf2dfad8a5cd900148f083f1208816	no	3
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20	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2054_-_Alexion_Press_Release_9-20-2006.pdf	71842 18dae26092be97e39c51dfa54a880193f6cda586	no	2
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21	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2055_-_Alexion_Press_Release_9-26-2006_1.pdf	73211 81af90f0eb40fbae993424570bf78f229c18c982	no	2
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22	Non Patent Literature	AXJ-114BUSCN5_-_Alexions_Soliris_Receives_2008_Prix_Galien_USA_Award_for_Best.pdf	66186 4e71f4a03ce2f52a4e1b33c4c94953e164d07599	no	2
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23	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2088_-_Amgen_Biosimilar_Pipeline_2.pdf	62267 3275edb428b65e3f7b20e5e646d8cfcfeed08a0f	no	2
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24	Non Patent Literature	AXJ-114BUSCN5_-_2019-12-02_504_Objections_to_Evidence_IPR201900739.pdf	230530 a6888f6f42510c4a62ea81bad3f1fc396a688cc3	no	9
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25	Non Patent Literature	AXJ-114BUSCN5_-_2019-12-02_880_Objections_to_Evidence_IPR201900740.pdf	230573 4d5392d2c69aff406215f594c6bcd97fc65639e1	no	9
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26	Non Patent Literature	AXJ-114BUSCN5_-_2019-12-02_149_Objections_to_Evidence_IPR201900741.pdf	230182 b061433d432b46f70138a19e92852b73ca154866	no	9
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27	Non Patent Literature	IPR201900739_Patent_Owners_Response.pdf	1690039 7ea040a0c1cdb66ae6cf30edf6b023571eeab60d	no	80
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Information:					

28	Non Patent Literature	IPR2019-00740_Patent_Owners_Response.pdf	1506654 2837ec9a84d2f62f67be269784e2cfd4eae90f93	no	80
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29	Non Patent Literature	IPR21900741_Patent_Owners_Response.pdf	1814396 15c3b164b89b391d1f2945ebe4bb047ac72bd807	no	83
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30	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2043_-_Andersen_2004.pdf	8382448 5f157431c6acf43886c6c31f5953a821bc38f11b	no	7
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31	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2078_-_ATCC_Packing_Slip.pdf	1576517 253fa9df35dc5fac6822e98b8c408d2ed36fce26	no	1
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32	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2074_-_Product_Sheet_HB-11625.pdf	132497 25901e4399b20d158d7a57d0b40aa2a9952514b8	no	2
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33	Non Patent Literature	AXJ-114BUSCN5_-_BusinessWire_Alexions_Soliris_Receives_2009_Prix.pdf	180244 dabfaf171129479 1f559f7e02c3e38f735340c9c	no	3
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34	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2044_-_Chadd_2001.pdf	5384184 0676a5e6fe31b06d7386d5c8a7d767bf20309282	no	7
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35	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2077-_Chain_of_Custody_Log.pdf	1096954 ecec350735ca00feeeea42b411accd56371aa278	no	1
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36	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2079_-_Chang_2002.pdf	1938421 4a93e636bcf3818a8953b0e2cc2316999478 31a5	no	32
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41	Non Patent Literature	AXJ-114BUSCN5_-_Declaration_of_Bernhardt_L_Trout_PhD.pdf	1314674 cb634e7bd28296344f6269177a750fcef6e0aa7c	no	95
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42	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2056_BAZARKO_DECLARATION_880.pdf	258119 9ccd3f08da49cc5752546e98ace5824b435498a8	no	7
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43	Non Patent Literature	AXJ-114BUSCN5_-_Declaration_of_Michel_C_Nussenzweig_MD_PhD.pdf	1606718 446cdbc0409fce8350313ec33c6b5af99bc9b5ef	no	55
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44	Non Patent Literature	AXJ-114BUSCN5_-_Deposition_Transcript_of_Joseph_P_Balthasar_PhD_Amgen_Inc_v_Alexio.pdf	3682348 1a1b13c487e08ec8da295052517ab4787c2a0fce	no	342
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46	Non Patent Literature	AXJ-114BUSCN5_-_110016326_RESPONSE_AMGEN.pdf	2725165 0ba2cc7227dae1b8aba12bda584ac0885f1c2f9f	no	47
Warnings:					
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47	Non Patent Literature	AXJ-114BUSCN5_-_Declaration_of_Dr_Bruce_Andrien.pdf	9783358 9fbd3e4a6deee82cc6a7d9acd7f8270563b9b4b3	no	23
Warnings:					
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48	Non Patent Literature	AXJ-114BUSCN5_-_Declaration_of_Dr_Laural_Boone.pdf	2975314 fa639b86ed1eb083168ae6adea96c4f429621e3f	no	8
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49	Non Patent Literature	AXJ-114BUSCN5_-_Declaration_of_Dr_Leonard_Bell.pdf	1439250 2883aed2c061bc666d089c8a16eefce3b7721439	no	4
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50	Non Patent Literature	AXJ-114BUSCN5_-_DECLARATION_TOM_VINK.pdf	762461 e8f11a3b3d3170df34cf24e8ea468bff4dacc708	no	17
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51	Non Patent Literature	AXJ-114BUSCN5_-_Denosumab_Drug_Bank_accessed_November_04_2019.pdf	326711 f8dba94f3729796bc336af2201646e66a183cd85	no	6
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54	Non Patent Literature	AXJ-114BUSCN5_-_Ranibizumab_Drug_Bank_accessed_November_05_2019.pdf	306848 9214e3e4886844664e46b56dab3692d00bdebd32	no	6
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57	Non Patent Literature	AXJ-114BUSCN5_-_Third_declaration_of_Dr_Dino_Miano.pdf	563019 72c9dfb19145fce74d4e3795b1c625784637ebc5	no	6
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(Not for submission under 37 CFR 1.99)

Application Number	15642096
Filing Date	2017-07-05
First Named Inventor	Leonard BELL
Art Unit	1644
Examiner Name	J. L. Rogers
Attorney Docket Number	AXJ-114BUSCN5

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	Art Unit	1644
	Examiner Name	J. L. Rogers
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	33	Chain of Custody Log, 1 page (2019).	
	34	Chang, B. et al., "Practical Approaches to Protein Formulation Development," Pharmaceutical Biotechnology, vol. 13:1-25 (2002).	
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	38	Declaration of Arturo Casadevall and Curriculum Vitae, dated November 22, 2019, 251 pages.	
	39	Declaration of Bernhardt L. Trout, Ph.D. and Curriculum Vitae, dated November 22, 2019, 95 pages.	
	40	Declaration of Daniel Bazarko, dated November 22, 2019, 7 pages.	
	41	Declaration of Michel C. Nussenzweig, M.D., Ph.D. and Curriculum Vitae, dated November 21, 2019, 55 pages.	
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	15642096
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	First Named Inventor	Leonard BELL
	Art Unit	1644
	Examiner Name	J. L. Rogers
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	44	European Application No. 11001632.6 Amgen's Response to the Statement of Grounds of Appeal filed by the Appellant on June 24, 2019, dated November 8, 2019, 47 pages.	
	45	European Application No. 11001632.6 Declaration of Dr. Bruce Andrien, dated May 23, 2017, 23 pages, cited in Opposition.	
	46	European Application No. 11001632.6 Declaration of Dr. Laural Boone, dated May 11, 2017, 8 pages, cited in Opposition.	
	47	European Application No. 11001632.6 Declaration of Dr. Leonard Bell, dated January 12, 2012, 4 pages, cited in Opposition.	
	48	European Application No. 11001632.6 Declaration of Tom Vink, dated August 6, 2009, 17 pages, cited in Opposition.	
	49	European Application No. 11001632.6 Denosumab, Drug Bank, accessed November 04, 2019, 6 pages, cited in Opposition.	
	50	European Application No. 11001632.6 European Board of Appeals Decision, File number T 386/89, dated March 24, 1992, 16 pages, cited in Opposition.	

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A point mutation in the CH3 domain of human IgG3 inhibits antibody secretion without affecting antigen specificity

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Abstract

Immunoglobulins (Ig) require correct folding and assembly of both heavy (H) and light (L) chains to form a functional H_2L_2 dimer that is secreted from plasma cells. This process is dependent upon the endoplasmic reticulum (ER) chaperone BiP, which targets improperly, folded or assembled Ig molecules for degradation. While investigating the mechanism of low IgG3 secretion, we identified a missense mutation L368P in the CH3 region of the human γ3 H-chain that was associated with impaired secretion of intact and functional Ig. The non-secreted H-chains displayed slower electrophoretic migration than secreted H-chains, consistent with them being glycosylated in the ER but not fully processed in the golgi apparatus and secretory pathway. Reversion of the mutated codon to wild type restored secretion of the IgG3, which displayed the same fine specificity for antigen as non-secreted IgG3. However, the non-secreted IgG3 was not opsonic in an in vitro phagocytosis assay. The results indicate that correct IgG3 CH3 domain folding is essential for secretion and effective function but does not affect specificity for antigen.
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Keywords: Recombinant immunoglobulin; Chimeric IgG3; Secretion defect; *Cryptococcus neoformans*; Punctate immunofluorescence

1. Introduction

The technology and methodology for the production of monoclonal antibodies has progressed at a remarkably fast pace since the original description of the hybridoma technique (Kohler and Milstein, 1975). It is now possible to utilize recombinant DNA technology to produce monoclonal antibodies with desired specificities from mammalian cells as well as bacteria and yeast, and in transgenic animals and plants (Anon., 1999). All of these methods still rely on the ability of the mammalian immune system to create antibody specificity but use quite different techniques to identify and produce specific antibodies.

The difficulties, both technical and inherent, in adapting the hybridoma strategy to the production of human monoclonal antibodies, particularly those targeting human proteins, has led to the adoption of protein engineering techniques for reducing the immunogenicity of murine monoclonal antibodies to the human immune system. This necessity of the use of recombinant DNA techniques to modify monoclonal antibodies has led to the production of antibodies from transfected mammalian cells, becoming the most common source of therapeutic antibodies. Other strategies for generating human monoclonal antibodies, such as SLAM

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or phage display, also require systems for the production of the recombinant immunoglobulins (Ig). For example, once the B-lymphocyte producing the antibody of desired specificity is identified, cloning of the relevant heavy (H) and light (L) chain Ig genes enables the unlimited production of monoclonal antibodies of the same specificity (Babcook et al., 1986). With this method, the rearranged Ig variable region genes (VH and VL) of choice that were amplified from selected antibody forming cells can be used in immunoglobulin expression vectors that encode functional Ig of any class. Likewise phage display strategies generate VH and VL genes that must be expressed as Ig for many applications (Barbas et al., 2001). Plasmid expression vectors have been described that contain constant (C) region genes for many of the human Ig isotypes (Coloma et al., 1992; Norderhaug et al., 1997; McLean et al., 2000) and the methods for the introduction of such vectors into mammalian cells and for harvesting of antibodies from culture supernatants are well established. However, despite the power of this technology, the production of antibodies in this way can be problematic. The recombinant antibodies are generally secreted at lower rates than traditional hybridomas, thus reducing the overall yield. These difficulties may be overcome by the use of alternative expression systems such as bacteria and insect cells but these bring additional complications, such as alterations in post-translational processing of proteins. Furthermore, loss of secretion or alterations in biological activity due to potential incompatibility of the V and C regions can also occur, highlighting the delicate nature of this technique (Yoo et al., 2002).

Previously we synthesized a matched set of Ig expression vectors designed to produce fully human, fully murine or chimeric recombinant antibodies from mammalian cells (McLean et al., 2000). All of these vectors, except the vector used for the production of human or chimeric IgG3, produced functional, secreted Ig molecules. Recombinant chimeric mouse–human IgG3 (chIgG3) was detected at only very low levels in the culture supernatant, although chIgG3 that retained antigen specificity could be isolated from cell-lysates (McLean et al., 2002). Analysis of the IgG3 C region of this vector revealed two nucleotide differences from what was expected from the published germ-line sequence. Here we describe that mutation of these two nucleotides to the published sequence resulted in secretion of chIgG3 molecules from this expression vector, suggesting that our original sequence had been affected by PCR-induced errors during construction. Our results provide some insight into the effects on secretion of recombinant antibodies through protein folding.

2. Materials and methods

2.1. Immunoglobulin expression vectors and site-directed mutagenesis

Expression vectors (pLC-huC$_\kappa$, pHC-huC$_\gamma$1, pHC-huC$_\gamma$3) used for the production of chIgG1 and chIgG3 were exactly the same as described previously (McLean et al., 2000). The Ig V domains for the murine monoclonal antibody 18B7 (Casadevall et al., 1998) were used to construct mouse–human chIgG1 and chIgG3; these sequences have been described previously (McLean et al., 2002). To alter Pro 136 and Pro 368 residues in the human C$_\gamma$3 sequence (GenBank accession number AJ294732), site-directed mutagenesis was performed on the pHC-huC$_\gamma$3 plasmid. The method was based on the Quik-Change protocol using high fidelity Pfu polymerase (Stratagene, Carlsbad CA). Briefly, PCR was performed using pHC-huC$_\gamma$3 as a template with the following conditions 95 °C for 30 s, 55 °C for 30 s, and 68 °C for 14 min for 16 cycles. Primers for converting Pro 136 to Ser were; ccaggagcacc**t**ctgggggcacag and ctgtgcccccag**a**ggtgctcctgg, and for converting Pro 368 to Leu were; gcctgacctgcc**t**ggtcaaaggcttc and gaagcctttgacc**a**ggcaggtcaggc (altered nucleotides are shown in bold). Successful mutation of codons encoding Pro 136 to Ser and Pro 368 to Leu in the plasmids was confirmed by bi-directional sequencing.

2.2. Production and purification of chimeric IgG

HEK 293 cells were transiently transfected with plasmids encoding L-chain and various H-chains to produce chIgG1 and chIgG3. Briefly, 3 μg of each plasmid was mixed with lipofectamine reagent (Invitrogen, Carlsbad, CA) and added to a monolayer of 4×10^6 HEK 293 cells for 6 h at 37 °C. Complete media (DMEM including 10% FCS) was then added, harvested and re-added every 24 h for up to 5 days. To harvest non-secreted chimeric IgG3, cells were collected by centrifugation at 24 and 48 h post-transfection and lysates were made using PBS containing 1% IGEPAL (Sigma, St. Louis, MO). Recombinant chIgG1 and chIgG3 were purified from either cell supernatants or cell-lysates using a protein-G affinity column (Amersham Biosciences, Uppsala, Sweden). Following elution with 100 mM glycine pH 2.5, the purified chIgG was dialyzed over 2 days versus three changes of PBS. Preparations were quantitated for IgG level by BCA protein assay (Pierce Biotechnology, Rockford, IL).

2.3. ELISA

This was performed as previously described for both GXM binding and chIgG production (McLean et al., 2002). Intact chIgG was assayed by capture with anti-L-chain antibodies and detected by using anti-H-chain antibodies. Anti-human γ chain, anti-human κ chain, both unlabelled and conjugated to alkaline phosphatase were from Southern Biotechnology Associates (Birmingham, AL).

2.4. Immunoblotting

Lysates of transfected 293 cells were prepared using RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate, 0.1% SDS) containing protease

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inhibitors (Complete Mini, EDTA-free) (Roche Diagnostics GmbH, Mannheim, Germany) and the protein content quantified by BCA protein assay (Pierce Biotechnology, Rockford, IL). For secretors 30 μg, and for non-secretors 75 μg of whole cell lysate was separated by SDS-PAGE (12%), transferred to nitrocellulose membranes, and analyzed by blotting with anti-human γ chain conjugated to peroxidase (Southern Biotechnology Associates, Birmingham, AL).

2.5. Immunofluorescence staining

Transfected 293 cells were fixed in 4% paraformaldehyde, blocked, and permeabilized in PBS containing 4% FCS and 0.2% Triton-X. Anti-human golgin-97 (Molecular Probes, Eugene, OR) was used for Golgi staining. Alexa 488-conjugated (anti-mouse IgG) (Molecular Probes, Eugene, OR) or Texas red-conjugated (anti-human IgG) (Southern Biotechnology Associates, Birmingham, AL) secondary antibodies were used according to the recommendations of the manufacturer. Images were acquired and analyzed as described (Noseda et al., 2004).

2.6. Cryptococcal assays

Both the immunofluorescence (IF) staining and phagocytosis assays were performed as described previously (McLean et al., 2002). Enriched chIgG preparations from supernatants or cell-lysates were used for IF staining whilst protein-G purified chIgG3 was used for the phagocytosis assay.

2.7. Homology modelling of human IgG3 Fc region

The amino acid sequence of human IgG3 was aligned with sequences of proteins deposited in the Protein Data Bank (PDB) using BLAST (http://www.ncbi.nlm.gov/BLAST/). PDB entry 1HZH (human anti-HIV B12 antibody; Saphire et al., 2001) was identified as a protein with 94% amino acid sequence identity to IgG3 in the Fc region. WT IgG3 and L368P IgG3 amino acid sequences were used as target sequences. The coordinates of the 1HZH_B chain and 1HZH_K chain were used as templates to generate a dimeric model with the help of the web-based Swiss-Model software (Peitsch, 1995; Guex and Peitsch, 1997; Schwede et al., 2003). The results are displayed using PyMol graphic software (DeLano Scientific LLC, San Carlos CA, USA http://www.pymol.org).

3. Results

3.1. Human IgG3 constant region cDNA clones

The single clone of human IgG3 C region cDNA (IGHG3) we had previously amplified by PCR contained two nucleotide differences from the closest IGHG3 sequence in nucleotide databases. Our IGHG3 cDNA had been amplified from cells expressing a mouse–human chIgG3 antibody (Sandlie et al., 1989) and has been described previously as part of the Ig expression vector pHC-huC$_\gamma$3 (McLean et al., 2000). Thus, nucleotides 59 and 893 of the C region cDNA were both found to be c instead of t, resulting in predicted amino acid substitutions of Ser to Pro in CH1 and Leu to Pro in CH3, at positions 136 and 368 (Eu numbering; Edelman et al., 1969), respectively. This sequence (GenBank accession number AJ294732) was closest to that of IGHG3*01, containing exons for CH1, CH2 and CH3 as well as four hinge exons (GenBank accession number X03604) (Huck et al., 1986), one of the 19 alleles displayed in the IMGT database (http://imgt.cines.fr) (Lefranc et al., 1999). Recombinant Ig molecules expressed using our human C region were not secreted from the cells. Defective secretion was also observed when the chIgG3 H-chains were co-expressed with either murine κ chains (McLean et al., 2000) or chimeric mouse–human κ chains (McLean et al., 2002), although functional Ig was isolated from cell-lysates in the latter case. The associated VH region was not the cause of the secretion defect, since a similar failure of secretion was observed when the C$_\gamma$3 region was expressed together with two different murine VH domains, R45 and 18B7. Furthermore, the secretion defect was not cell type specific since we observed a lack of secreted chIgG3 from both a murine plasmacytoma cell line (NS0) and human embryonic kidney cells (HEK 293).

All human IgG isotypes (IgG1, IgG2, IgG3, and IgG4) contain Ser at position 136 and Leu at position 368, suggesting the importance of these residues in the protein structure. We suspected that the substitution of Pro at these positions in our IgG3 molecules were responsible for the absence of Ig secretion. To explore this hypothesis, we altered these nucleotide differences in our IGHG3 cDNA sequence of pHC-huC$_\gamma$3 and evaluated their effect on the secretion of chIgG3 molecules. Site-directed mutagenesis was performed to revert the nucleotide sequence to the sequence of allele IGHG3*01. We constructed three new IGHG3 cDNA sequences and thus, pHC-huC$_\gamma$3 plasmids, with either or both of the Pro residues changed to the Ser or Leu present in IGHG3*01 (Fig. 1). All pHC-huC$_\gamma$3 constructs contained the VH domain of the murine monoclonal antibody 18B7 (GenBank accession number AJ309276) specific for *Cryptococcus neoformans* capsule glucuronoxylomannan (GXM) (Casadevall et al., 1998).

3.2. Secretion analysis of chIgG3 molecules

The various pHC-huC$_\gamma$3-18B7VH constructs and the control pHC-huC$_\gamma$1-18B7VH construct were co-transfected with pLC-huC$_\kappa$-18B7V$_\kappa$ (18B7V$_\kappa$ GenBank accession number AJ309277, human C$_\kappa$ GenBank accession number AJ294735) into HEK 293 cells to monitor transient expression of chIgG3. Culture supernatants and cell-lysates were harvested at both 24 and 48 h following transfection. These Ig preparations were tested by ELISA for production of chIgG and for binding to GXM. The IgG3 secretion results are

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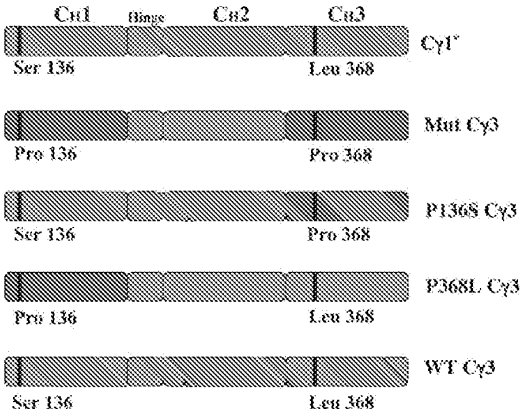


Fig. 1. Schematic diagram of constant regions for the H-chain chIgG clones. Mutated (P136, P368) and wild type (S136, L368) residues are indicated at the corresponding positions of each C_γ region. Darker shading represents a domain with a mutated residue. * The human $C_\gamma 1$ differs from human $C_\gamma 3$ in that the hinge region is shorter but is not displayed as such in this figure.

shown in Fig. 2. Control chIgG1 was efficiently secreted into the supernatant at both 24 and 48 h following transfection whereas the original chIgG3 (Mut IgG3) was not secreted although it was detectable in cell-lysates at 24 h post-transfection (Fig. 2A and B), consistent with previous results. This confirms that the Mut IgG3 H-chains were produced by the transfected cells and were paired with the chimeric L-chains but that the Ig molecules were not secreted from cells despite the presence of identical heavy chain Ig leader sequences, VH regions and L-chains. chIgG3 expressed with just the CH1 Pro residue reverted to wild type Ser (P136S IgG3), behaved like Mut IgG3 and were not secreted, whereas chIgG3 expressed with the CH3 Pro residue reverted to wild type Leu (P368L IgG3) or both Pro residues which reverted to the canonical (WT IgG3) were both efficiently secreted from cells (Fig. 2A and B). Calculations of the ratio of secreted IgG to internal IgG from these cells confirm an approximate 1:1 ratio for secretors which is reduced by 10- to 20-fold for non-secretors (Table 1). These results support the contention

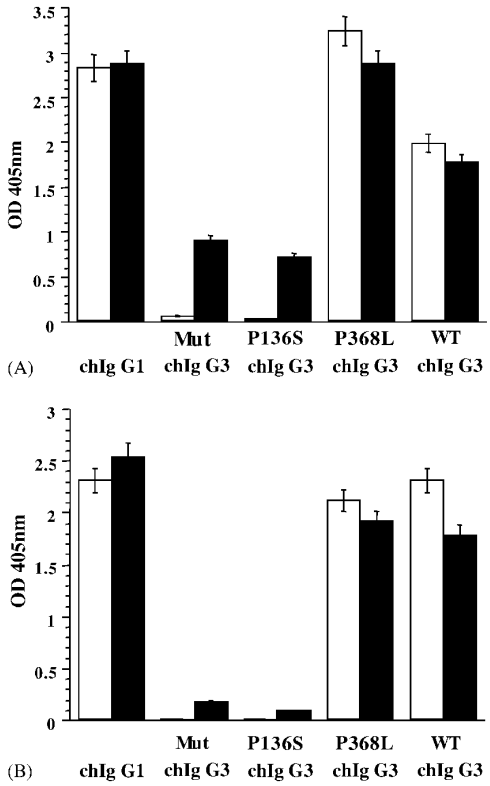


Fig. 2. chIgG3 with the L368P mutation are not secreted from cells. Supernatants (open columns) and cell-lysates (closed columns) of 293 cells, co-transfected with the indicated plasmid constructs encoding H-chains and L-chains for production of chIgG, were analyzed after (A) 24 h and (B) 48 h for chIgG production by capture ELISA.

that the presence of the Pro residue at position 368 in CH3 of human IgG3 negatively affected secretion of these proteins while the Pro residue at position 136 of CH1 had no such adverse effect on protein secretion.

To ascertain whether the chimeric $C_\gamma 3$ H-chains produced by transfected cells had the expected molecular mass, we

Table 1
Analysis of anti-*C. neoformans* chIgG secretion and function

chIgG clone	Amino acid		Secreted/internal IgG index[a]	GXM ELISA[b]	*C.neoformans* IF pattern	Phagocytosis index[c]
	@ 136	@ 368				
IgG1	Ser	Leu	0.98	++	Annular[d]	+++[d]
Mut IgG3	Pro	Pro	0.07	++	Punctate	+/−
P136S IgG3	Ser	Pro	0.04	+++	nd[e]	+/−
P368L IgG3	Pro	Leu	1.13	++++	Punctate	++
WT IgG3	Ser	Leu	1.12	++++	Punctate	++

[a] Data are summarized from Fig. 2. IgG was harvested 24 h post-transfection and diluted 1:4 for ELISA analysis. Results are expressed as the ratio of OD 405 nm obtained for secreted IgG/internal IgG.

[b] Data are summarized from Fig. 4. IgG was protein-G purified and normalized for protein content before ELISA analysis for GXM binding. The OD 405 nm values achieved with 1 μg/ml chIgG were assigned as follows: 0–0.2 +, 0.2–0.4 ++, 0.4–0.6 +++, 0.6–0.8 ++++.

[c] The ability of chIgG to opsonize *C. neoformans* was measured as previously described (McLean et al., 2002). Phagocytosis index is assigned as follows: no opsonization −, weak opsonization +, medium opsonization ++, strong opsonization +++.

[d] Data previously published (McLean et al., 2002).

[e] Not done.

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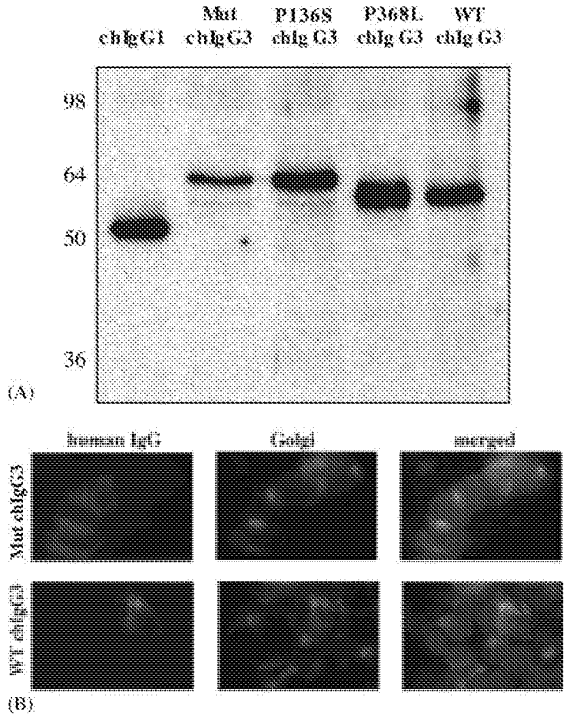


Fig. 3. Secreted and non-secreted chIgG3 H-chains have different molecular masses and localize to different subcellular compartments. (A) Whole cell-lysates were made 24 h after co-transfection with the indicated plasmid constructs encoding H-chains and L-chains for production of chIgG. H-chains were assessed by immunoblot with anti-human γ chain. M: molecular mass. (B) Immunofluorescence staining of HEK 293 cells transfected to express either Mut chIgG3 (upper panels) or WT chIgG3 (lower panels) was performed using anti-human γ chain or anti-human golgin-97. Merged images with Dapi staining of nuclei are shown at the far right.

analyzed cell-lysates by western blotting. Interestingly, we found that the mobility of the non-secreted H-chains was slower than that of secreted H-chains despite almost identical molecular weight predicted for the protein product from the cDNA (Fig. 3A). Thus, the electrophoretic molecular weight for the non-secreted H-chains (Mut and P136S), secreted H-chains (P368L and WT) and control $C_\gamma 1$ H-chain was ~60, ~57, and ~52 kDa, respectively. The most straightforward interpretation of these results is that the non-secreted $C_\gamma 3$ chains were glycosylated differently than the secreted $C_\gamma 3$ chains. To confirm our suspicion that the increased molecular weight of non-secreted H-chains was the result of unprocessed N-linked carbohydrate chains due to blockade early in the secretory pathway, we performed IF staining of transfected 293 cells to compare the localization of secreted and non-secreted H-chains (Fig. 3B). We found that both non-secreted and secreted H-chains were expressed throughout the cytoplasm whilst secreted H-chains tended to co-localize with the golgi apparatus marker, unlike the non-secreted H-chains (Fig. 3B, lower right panel). This result is consistent with the non-secreted H-chains being incorrectly folded and unable to traffic to the golgi, hindering their passage through the secretory pathway.

3.3. Reactivity of chIgG3 with GXM

We next evaluated the functionality of the chIgG by testing their binding to the cognate antigen GXM. To facilitate the analysis of GXM binding by all the chIgG clones, the Ig's were purified from supernatants and cell-lysates by protein-G chromatography. Preparations of purified IgG were normalized for protein content and tested for binding to GXM by ELISA. Here, significant binding of both the secreted and non-secreted chIgG3, as well as the control chIgG1 was observed (Fig. 4). Taken together, these results confirm that chIgG3 with the described amino acid differences in either CH1 or CH3, that have opposing effects on protein secretion, are still bound to GXM in ELISA experiments with characteristics similar to those of canonical chIgG3 and chIgG1.

3.4. Immunofluorescence patterns of chIgG3 with C. neoformans

Since all of the purified chIgG3 preparations were bound to GXM by ELISA, we decided to re-evaluate the fine specificity of binding to *C. neoformans* cells by immunofluorescence (IF). We have previously shown that the fine specificity of antibodies, expressing mAb 18B7 V regions, for the polysaccharide capsule of *C. neoformans* differed depending on the antibody isotype (McLean et al., 2002). In this system, differences in IF pattern produced by antibody binding to the polysaccharide capsule reflect differences in antibody fine specificity and two major patterns known as annular or punctate have been described. Thus, the murine mAb 18B7 (IgG1-κ) and chIgG1, chIgG2, chIgG4, each bound in an annular pattern to the *C. neoformans* capsule, whereas chIgG3, with identical 18B7 VH and V_κ region sequences, produced a punctate pattern. This result was interpreted as indicative of C region induced changes in fine specificity of antigen

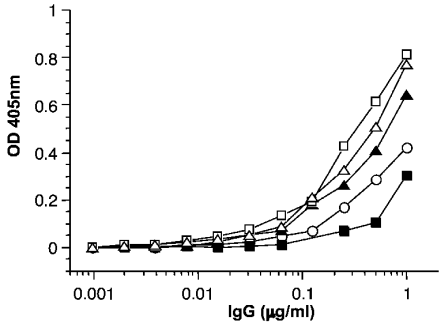


Fig. 4. Secreted and non-secreted chIgG3 bind to GXM. Direct ELISA to *C. neofomans* GXM with protein-G purified chIgG. The various chIgG are designated as follows. Secreted chIgG (open symbols), chIgG1 (circle), P368L chIgG3 (square), WT chIgG3 (triangle). Non-secreted chIgG (closed symbols), Mut chIgG3 (square), P136S chIgG3 (triangle).

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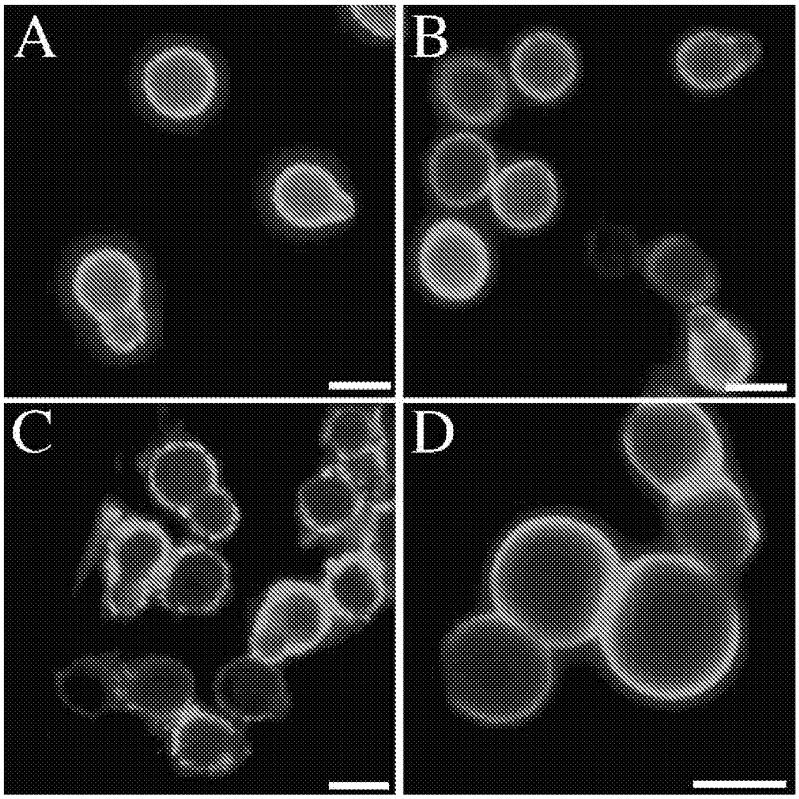


Fig. 5. Secreted and non-secreted chIgG3 immunofluorescence staining of *C. neoformans* cells is punctate. *C. neoformans* cell suspensions were incubated with secreted ((A) P368L chIgG3; (B) WT chIgG3) and non-secreted ((C), Mut chIgG3) chIgG3 antibodies and detected as previously described (McLean et al., 2002). Control staining with chIgG1 was done to display annular IF pattern for comparison (D). The scale bar represents 5 μm.

binding. However, the finding that the IgG3 C region contained two point mutations, that impeded secretion and normal glycosylation, raised the possibility that abnormal IgG3 structure may have been responsible for punctate staining. However, we observed here that antibodies with either our original IgG3 C region or the canonical one produced punctate IF patterns, demonstrating that the Pro residues were not responsible for the altered specificity relative to other isotypes (Fig. 5). Thus, secreted chIgG3 where Pro 368 was reverted to Leu 368 (P368L IgG3 and WT IgG3), both produced punctate IF (Fig. 5A and B) and the non-secreted chIgG3 clone (Mut IgG3), when purified from cell-lysates, also produced punctate IF (Fig. 5C) confirming the previously reported results with non-secreted chIgG3 (McLean et al., 2002). Importantly, the chIgG1 produced the expected annular IF (Fig. 5D). These results are consistent with our previously reported novel finding that antibody fine specificity to a polysaccharide antigen depends on the associated C region (McLean et al., 2002).

3.5. Opsonic efficacy of chIgG3

We assessed the ability of the various chIgG3 proteins to opsonize *C. neoformans*. Opsonic ability correlated with secretion efficiency. Thus, the secreted chIgG3 clones (P368L IgG3 and WT IgG3) and control chIgG1 showed significant opsonic efficacy, whereas the non-secreted chIgG3 (Mut IgG3 and P136S IgG3) were not opsonic (Table 1). These results are consistent with the hypothesis that the correct folding and/or glycosylation of the chIgG3 CH3 domain are required not only for secretion from cells but also for opsonic efficiency.

3.6. Human IgG3 Fc region model

We performed homology modeling of the canonical and the original chIgG3 Fc domain to pinpoint the contribution of Pro 368 to the non-secretion phenotype (Fig. 6). The relative positions of the N-linked glycosylation site of CH2 (Asn 297) and the Tyr 407 and Pro 368 residues in the CH3 dimer interface are indicated (Fig. 6A). Closer inspection of the CH3 dimer interface (Fig. 6B and C) reveals that the sidechains of Leu 368 form a close interaction with the sidechains of Tyr 407. One possible reason for the lack of secretion of chIgG3 containing Pro 368 is that this residue contributes to a lack of dimeric domain stability. Our model supports this possibility by predicting that the substitution of Pro 368 resulted in a

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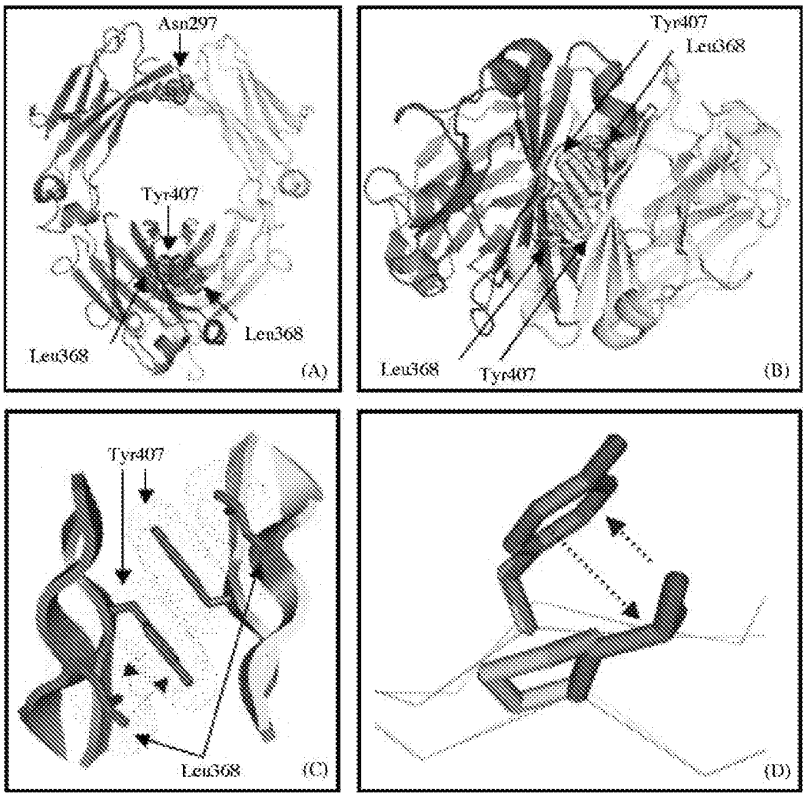


Fig. 6. Structural analysis of IgG3 Fc region and L368P mutant by homology modeling. (A) View perpendicular to the 2-fold rotation axis between the H-chain dimer interface of WT IgG3 Fc region. The locations of residues Asn297 (glycosylation site), Leu368 (mutant residue), and Tyr407 (structurally important) from each monomer are displayed in red with the van der Waals surfaces of their side-chains represented by dots. (B) View along the 2-fold rotation axis between the H-chain Fc dimer looking down the interface. Residues Leu368 and Tyr407 from each monomer are displayed in red with the van der Waals surfaces of their sidechains represented by dots. (C) Blow up of Tyr407 stacking and Leu368 clamp. (D) A close-up of WT model backbone (magenta) and the L368P model backbone (cyan). The sidechains of residues Leu368 and Pro368 as well as Tyr407 are displayed and indicate the loss of "clamping" on Tyr407 caused by the L368P mutation.

lack of clamping to the stacking interaction of Tyr 407 at the interface between the two CH3 monomers (Fig. 6D). This clamping would normally be provided by the sidechain of Leu 368. Lower stability of this domain, due to the presence of Pro 368, may block entry of the proteins into the secretory pathway from the ER, where they remain destined for degradation.

4. Discussion

Prior studies had shown that our pHC-huC$_\gamma$3 expression vector produced intact chIgG3 that remained within the cell and was not secreted (McLean et al., 2002). To investigate this problem we analyzed the C$_\gamma$3 plasmid sequence and noted two nucleotide differences from the closest human C$_\gamma$3 sequence in the database that resulted in replacement amino acid substitutions. We considered the possibility that our mutations represented polymorphisms or that perhaps the database sequence was in error. Establishing the correct sequence was important because one or both of these amino acid differences could be responsible for the impaired secretion observed with this vector. Furthermore, the observation complicated interpretation of our finding that chIgG3 anti-cryptococcal antibodies had different fine specificity than other isotypes with identical V regions (McLean et al., 2002). Thus it was conceivable that the alteration in pattern of IF was a consequence of the C region mutation and did not reflect a general property of chIgG3. Therefore, we analyzed the effect of these two amino acid differences on secretion and on IF pattern. Restoration of the nucleotides in our original construct to those in the canonical sequence resulted in a marked increase in the amount of secreted chIgG3, and corrected the secretion defect associated with the original pHC-huC$_\gamma$3 expression vector.

Our observations showed that the CH1 domain mutation Pro 136 was not associated with defective secretion. In contrast, the Pro 368 mutation in CH3 greatly reduced chIgG3 secretion. This result raised the possibility that the structure of the CH3 domain was affected by Pro 368 but the CH1 domain

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structure was not affected by Pro 136. Homology modelling of the original and canonical IgG3 Fc domain helped us to determine, in a structural context, the contribution of Pro 368 to the non-secretion phenotype. The model predicts that the presence of Pro 368 destabilizes the CH3 dimer interface via loss of interaction with important stacking Tyr 407 residues from each H-chain. This destabilized domain structure would be identified by ER chaperones, such as BiP, leading to the block of chIgG3 export and subsequent degradation within the ER.

Glycosylation has been implicated in the proper assembly and secretion of IgG molecules (Taylor and Wall, 1988) and human IgG3 contains a biantennary complex N-linked carbohydrate at residue Asn 297 within CH2 (Rademacher et al., 1986). We noted for the non-secreted chIgG3 H-chains differences in electrophoretic migration compared to that of the secreted chIgG3, suggesting that the non-secreted H-chains due to CH3 instability were retained in the ER, preserving the initial high mannose and unprocessed carbohydrate side chains. In fact, our experiments showed that the non-secreted H-chains could not localize to the golgi apparatus, unlike secreted H-chains. This is supported by the recent demonstration that a recombinant IgG with VH-associated N-linked carbohydrate side chains that remain high in mannose, is not secreted and is retained in the ER (Gala and Morrison, 2004).

Our results raised the possibility that the presence of not fully processed carbohydrate in the non-secreted chIgG3 isolated from within cells could alter the ability of these antibodies to carry out effective function and/or alter their fine specificity for antigen. Interestingly, all chIgG3 bound efficiently to the purified cryptococcal capsular polysaccharide GXM, including the non-secreted chIgG3. Previously we had shown that the anti-cryptococcal chIgG3 isolated from cell-lysates, since it was not secreted, produced a punctate IF pattern (McLean et al., 2002). Prior work has established two major patterns of antibody binding to the *C. neoformans* polysaccharide capsule, annular and punctate, which are named based on their microscopic fluorescence appearance. Antibody 18B7 produces annular IF, so to determine whether this change in fine specificity was associated with the amino acid differences within the C region, we repeated the IF using the repaired chIgG3. When these chIgG3 were compared for binding to *C. neoformans* cells by IF, we noted that the staining pattern was punctate rather than the typical annular pattern previously described for mAb 18B7 (Casadevall et al., 1998). Our finding that repaired chIgG3 expressing the wild type sequence produced the same IF upon binding *C. neoformans* indicate that the changes in fine specificity for the non-secreted chIgG3, were not a result of alterations in primary C region sequence. These results are consistent with, and supportive of, the notion that V regions may differ in fine specificity when associated with different C regions (Cooper et al., 1993; Pritsch et al., 1996; McLean et al., 2002). Importantly, the CH1 mutation Pro 136, that did not inhibit secretion, also did not affect fine specificity for *C. neoformans*. We conclude that the presence of Pro at positions 136 and 368 in chIgG3 does not affect fine specificity for antigen whereas the Pro at position 368 affects chIgG secretion.

Non-secreted chIgG3 did not display effector function in an in vitro phagocytosis assay while secreted chIgG3 could opsonize *C. neoformans*, suggesting that, despite normal antigen specificity the instability and aberrant glycosylation of the Fc region may interfere with Fc receptor interaction and hence Ig effector function. In this regard, it is noteworthy that IgG C region N-glycan structures are critically important for the interaction of immunoglobulin with the Fc receptor (Mimura et al., 2001; Radaev et al., 2001). Furthermore, the Fc receptor-binding site of IgG is in the lower hinge and hinge-proximal region of CH2 (Jefferis and Lund, 2002), which is distant from the Pro 368 residue in CH3 but nearby the N-linked glycosylation site at Asn 297. An alternative explanation for the lack of opsonic ability of these chIgG3 preparations could simply be related to insufficient Ig present, since we could only purify relatively small amounts of the non-secreted chIgG3 protein and consequently could not perform a detailed dose–response evaluation. Further investigation with higher concentrations of the non-secreted chIgG3 will be required to demonstrate whether their apparent inability to opsonize *C. neoformans* is due to the loss of interaction with the Fc receptor or not. Previously, antibodies displaying annular IF pattern on *C. neoformans* had stronger opsonic activity than antibodies that produced punctate IF (Nussbaum et al., 1997; Cleare and Casadevall, 1998). The results of this study suggest that the association between punctate IF and non-opsonization is not ubiquitous in that some antibodies that produce punctate IF can opsonize equally as well as antibodies that produce annular IF.

An association has been made between annular IF and IgM-mediated phagocytosis in the absence of complement (Taborda and Casadevall, 2002). That phenomenon presumably involves a structural change in the capsule that promotes phagocytosis through the complement receptor by direct polysacchride-CD18 interactions (Taborda and Casadevall, 2002). In contrast, IgM mAb's that produce punctate IF are not opsonic by this mechanism, presumably because the necessary capsular structural changes that allow the interaction with the complement receptor does not occur. In this study we noted that the secreted chIgG3 was opsonic despite producing a punctate IF pattern on *C. neoformans* capsules. We interpret this phenomenon as indicative of the fact that chimeric IgG3, unlike IgM, can interact with Fc receptor and promote phagocytosis without engaging complement receptors. Hence, the association between punctate binding and lack of opsonic efficacy does not apply to antibodies that can engage receptors directly.

The findings presented herein describe the restoration of secretion of recombinant chIgG3 molecules from the expression vector pHC-huC$_\gamma$3. This was important for two reasons: (1) a reliable system for producing functional, secreted recombinant IgG3 was required and (2) to confirm that our previous observation that the alteration of fine specificity of 18B7-chIgG3 was not due to amino acid differences within

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the $C_\gamma 3$ sequence. We have achieved these aims and presented the results in this report. The implications of this work are enhanced by the fact that mAb 18B7 is currently in clinical evaluation and that mouse–human chimeric antibodies are increasingly being used in human therapy.

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	Art Unit	1644
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Signature	/Jill Gorny Sloper, Esq./	Date (YYYY-MM-DD)	2019-12-11
Name/Print	Jill Gorny Sloper, Esq.	Registration Number	60,760

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Electronic Acknowledgement Receipt

EFS ID:	37989322
Application Number:	15642096
International Application Number:	
Confirmation Number:	7606
Title of Invention:	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT
First Named Inventor/Applicant Name:	Leonard BELL
Customer Number:	133485
Filer:	Jill R. Gorny Sloper/Anita Costa
Filer Authorized By:	Jill R. Gorny Sloper
Attorney Docket Number:	AXJ-114BUSCN5
Receipt Date:	11-DEC-2019
Filing Date:	05-JUL-2017
Time Stamp:	16:20:55
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	Non Patent Literature	AXJ-114BUSCN5_-_Second_declaration_of_Dr_Dino_Miano.pdf	137151 c5d9e9dd0b687375ff3ab7a038ed9d26bca6920d	no	3

Warnings:

Information:					
2	Non Patent Literature	AXJ-114BUSCN5_-_European_Application_No_161723358_Third_Party_Observation_dated_Septem.pdf	283531 8549e607a5790e4a21759433fce3e8a3c06ac1de	no	5
Warnings:					
Information:					
3	Non Patent Literature	AXJ-114BUSCN5_-_European_Application_No_161723358_Third_Party_Observation__94.pdf	418946 62491a9f50524ba6b43fe12816a110971c4aa8bf	no	8
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4	Non Patent Literature	AXJ-114BUSCN5_-_European_Application_No_161953559_WRITTEN_SUBMISSION.pdf	1098475 a63a47db319ed61f0c684989ce1d4e54e2efd731	no	18
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5	Non Patent Literature	AXJ-114BUSCN5_-_European_Application_No_161953559_Third_Party_Observation.pdf	283377 533c028cbd34616e7d99743063716293e656554d	no	5
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6	Non Patent Literature	AXJ-114BUSCN5_-_European_Application_No_161953559Third_Party_Observation_dated_Septemb.pdf	437776 d99fe4ff88922b244b7212ae436173950680950a	no	8
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7	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2050_-_Fatimah.pdf	1107469 d468d3f50614ad72c9722f71cfdeda59828f58bf	no	10
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8	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2051_-_Foote_1992.pdf	1821051 b3a1491ff97de221ddbaa36040d5c40a7e87f0df	no	13
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9	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2082_-_Frokjaer.pdf	2413823 b2e3e9cbb392f453949247b8e8d355bcd4f8f01a	no	10
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10	Non Patent Literature	EXH_2089_-_Generium_Elizaria.pdf	86132 1e1abc0837d69615c1e79171d93b7d9c13beb5f7	no	2
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11	Non Patent Literature	EXH_2075_-_GenScript_Final_Report.pdf	368377 554917741e9634133e81eb8f56d4da4e6a8e6948	no	4
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12	Non Patent Literature	EXH_2076_-_GenScript_Sequencing_Record.pdf	890395 20c1c2af53ec55c7999834987baf52117b57c1d2	no	4
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13	Non Patent Literature	Project_Proposal_for_Monoclonal_Antibody_Sequencing_Service.pdf	1581380 8468ab2818ea75be10c0212ace828941470 2b746	no	3
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14	Non Patent Literature	AXJ-114BUSCN5_-_GREENSPAN.pdf	947396 2df3172aa35f4ed75e3c8f2bc2e56ab68de1c2ae	no	5
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15	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2046_-_1997_INN_Naming_Guidelines.pdf	2185545 e7f6d5cba47629dc1aa3d150abc66cfcceb8f6ad	no	41
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16	Non Patent Literature	AXJ-114BUSCN5_-_Haller_et_al_Safety_Issues_Specific_to_Clinical_Development.pdf	162987 7f6517c7b40e2cf2320ecd587d559bcfc8986df3	no	4
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17	Non Patent Literature	EXH_2091_-_Harlowe_and_Lane_.pdf	12118447 c56cc368ac440ba327c62e4650c6160ad57648ab	no	7
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18	Non Patent Literature	AXJ-114BUSCN5_-_HAWKINS.pdf	1740772 1ecfb2b9e57d41f23e76c39ab2601d40ad674941	no	7
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19	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2049__-_HemeOnc_Today.pdf	263796 26efb6cf82e58d8036fdc6b30140158c1acdc382	no	9
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20	Non Patent Literature	EXH_2081_-_Hermeling.pdf	848554 9e3baaa3814948884db2950c34fc62146748bf332	no	7
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21	Non Patent Literature	AXJ-114BUSCN5_-_Hill_et_al_Erythropoietin_treatment_during_complement_inhibition_with.pdf	599642 9736d4e0d44a4513074ce60458b9247ba1aa81c9	no	10
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22	Non Patent Literature	HILLMEN_Long-term_effect_of_the_complement_inhibitor_eculizumab.pdf	130493 2d32ee2748569fe4d9545962636ceb3a684ca2d7	no	7
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23	Non Patent Literature	HILLMEN_Long-term_safety_and_efficacy_of_sustained.pdf	212552 bcd944ee8b3a8394d6c11c50ffa9c2fff1299f60	no	12
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24	Non Patent Literature	HILLMEN-NATURAL_02.pdf	506472 6e620f15ac4518ae24b706c820a01d51e2c4e588	no	6
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25	Non Patent Literature	AXJ-114BUSCN5_-_Hwang_et_al_Immunogenicity_of_engineered_antibodies_SCIENCE.pdf	984680 09ba2ec90c7dc6220b6cd5ff3a74eeeef074aab9	no	8
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26	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2084_-_ICH_Topic_Q5C.pdf	209443 1383c2508c725d8d397286f97d990e3cc7162706	no	9
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27	Non Patent Literature	AXJ-114BUSCN5_-_JANDA.pdf	3052222 eb52c08023ab478ada533be4fd191751aaedf8d8	no	10
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28	Non Patent Literature	AXJ-114BUSCN5_-_Janeway_and_Travers_Immunobiology_The_Immune_System_in_Health_.pdf	2110503 fd5201652812ffe1372f0c25efb43210ab2f8898	no	12
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29	Non Patent Literature	AXJ-114BUSCN5_-_JANEWAY_Janeway_et_al_Immunobiology_The_Immune_System_in_Health_and.pdf	4716820 b064af599bbc9295d4c89fbfe39979d7a2afa84d	no	6
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30	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2053_-_Soliris_Current_Label_06-2019_Label.pdf	604553 d2ce79fd38528cb89202a9f36e1e82637805f010	no	35
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31	Non Patent Literature	KELLY.pdf	542857 9fc117f5f10731f4af57878af7917a6ac0550e91	no	7
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32	Non Patent Literature	AXJ-114BUSCN5_-_Kim_et_al_Antibody_Engineering_for_the_Development_of_therapeutic.pdf	1675535 1ad93fb38786ce3bd8117d9798b4e10c3f53933f	no	13
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33	Non Patent Literature	AXJ-114BUSCN5_-_Lo_Benny_K_Antibody_Engineering_Methods.pdf	12159204 38565b18081deb6aeea0944dbe05846c7f1ebb26	no	28
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34	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2042_-_Mathieu_2004.pdf	2373430 4d010694a92bd02b99a1e81e2e163e95e8219799	no	22
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37	Non Patent Literature	AXJ-114BUSCN5_-_Points_to_Consider_in_the_Manufacture_and_Testing_of_Monoclonal.pdf	796249 ed6dd8e2cdfa55a72ff6ffdc87c0952a3db1ddbf	no	50
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38	Non Patent Literature	AXJ-114BUSCN5_-_Pritsch_1996.pdf	255020 a1b9d1c1c9c07ccf53d8a99d9eeaa3a2b2842e7c	no	9
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39	Non Patent Literature	AXJ-114BUSCN5_-_PRITSCH_2000.pdf	1660282 ad25d83d2fcd22d9986a006a710d510ba389dec7	no	9
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40	Non Patent Literature	AXJ-114BUSCN5_-_RADBRUCH.pdf	460676 689d16816f197a391b37b9cb5322f30a28a0c354	no	3
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43	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2090_-_Samsung_Biosimilar_Pipeline.pdf	95398 9be24710167e8ec8e62961c9dbb1ca9a22f2bf12	no	3
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45	Non Patent Literature	EXH_2086_-_Stebbings_2013.pdf	2763908 d98c92f0c33ac400b3c1dadd1e1f28f789a3b9d7	no	8
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46	Non Patent Literature	TORRES_2007.PDF	1230473 5963c58123014d7d6655787109de8c07c67ce5a4	no	10
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49	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2085_-_Q1AR2_Stability_Testing_Guidance.pdf	302799 1fc7d4ee0a3e14b02fb62c872b47648dbfb5b5ef	no	25
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51	Non Patent Literature	AXJ-114BUSCN5_-_Welt_et_al_Phase_I_Study_of_Anticolon_Cancer.pdf	253276 12cf2444668757 4a4150739d59fb765afdb71351	no	10
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52	Non Patent Literature	AXJ-114BUSCN5_-_EXH_2052_-_Xiang_1995_1.pdf	217662 5690393f41fafd1e889d2a0c0bc668caa3a61428	no	6
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53	Non Patent Literature	SEC_8K_09052019.pdf	35919 aea61846f445dbb7c9b3d84c86bb33362eb73855	no	3
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54	Transmittal Letter	AXJ-114BUSCN5_-_IDSTRANS_02.pdf	27004 1d0c7ad1a51dda139d45f471f1afc595b5e886ca	no	2
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55	Information Disclosure Statement (IDS) Form (SB08)	SB02.pdf	1037615 5abd178a60357c453bc7818472605537cdb30757	no	8
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56	Information Disclosure Statement (IDS) Form (SB08)	SB01.pdf	1036390 d0c26f175aceee84e3f8fe67fe124561142287 83d	no	8
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57	Non Patent Literature	McClean_2005.pdf	936640 c5ed73830da82f66a0c043170d8ab82ceca15842	no	9

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58	Information Disclosure Statement (IDS) Form (SB08)	SB03.pdf	1035493 90ac5c762c0310f801906dcbb110c799a77dc546	no	5
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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

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IN THE MATTER OF
European Patent No. EP 2 359 834

SECOND DECLARATION OF DR. DINO MIANO

I, Dr. Dino Miano, hereby declare as follows:

1. I am Dr. Dino Miano of Alexion Pharmaceuticals, Inc., 100 College St, New Haven, Connecticut, USA. I am the same Dr. Dino Miano that submitted a declaration known as D33 in these proceedings. Details of my background and my CV were provided with that earlier declaration.

2. I understand that one of the Opponents in this opposition has indicated that no correlation has been shown between aggregation and loss of activity for eculizumab. I was therefore asked to comment on the activity of the aggregate (dimer) form of eculizumab.

3. In 2005 we carried out a number of analyses on the eculizumab dimer sample isolated from eculizumab drug product lot 5000-001A according to according to DVL-05-100-00, "Isolation of Eculizumab Dimer and Monomer." Dimer isolation was performed by manual collection of peaks eluting from a size exclusion column. Collected peaks were pooled and concentrated with a Centriplus YM-10 centrifugal filter device.

4. These analyses included a measure of the C5 binding assay of the dimer. The C5 binding assay determines potency by measuring the ability of eculizumab samples to bind to C5 proteins. The method used for the C5 binding assay was the BioVeris Detection Analysis of C5 binding to eculizumab (Alexion ref BTM-0018.01). The assay uses an electrochemiluminescent (ECLM) reaction and photon detection technology (measured at λ = 620 nm) to determine potency of eculizumab by testing the ability of an eculizumab sample to bind C5 proteins. A ruthenium ion, necessary for the ECLM reaction, is conjugated to C5, a human complement protein. The C5 protein and eculizumab sample are incubated in a microtiter plate. rProtein-L, a biotinylated protein, is added and incubated with the C5/eculizumab complex. After the incubation, magnetized streptavidin beads are added to the plate. The antibody complex attaches to the beads through a streptavidin-biotin association. After incubation with the beads, the sample plate is measured for ECLM detection with a BioVeris system.

5. The purity of drug product lot 5000-001A, as determined by size exclusion chromatography (SEC), was 98.8% monomer and 1.2% aggregate at release (T=0, 01JUN01). This lot was stored at 2-8°C for 52 months for use in this study. The purity after 52 months was determined to be 97.25% monomer and 2.75% aggregate. The aggregate was isolated as described in paragraph 3 above.

6. The aggregate (dimer) peak isolated from drug product lot 5000-001A contained

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approximately 95% dimer and 5% monomer when initially tested by SEC; refer to Figure 1.

7. The C5 binding assay was carried out according to the method described in paragraph 4 above for both eculizumab drug product lot 5000-001A and the isolated dimer sample. The source material, drug product lot 5000-001A, had a C5 binding capacity of 92.6%. The isolated dimer sample had roughly half the potency of lot 5000-001A, with a C5 binding capacity of 45.3%. A placebo (lot 2000-001A) was also tested and was below the limit of quantitation in this assay.

8. These results demonstrate that the isolated dimer has approximately 50% of the C5 binding activity of the drug product, indicating a significant drop in potency on aggregation to the dimer form.

I believe and do hereby declare that the facts stated above are to the best of my knowledge true.

Dr. Dino Miano

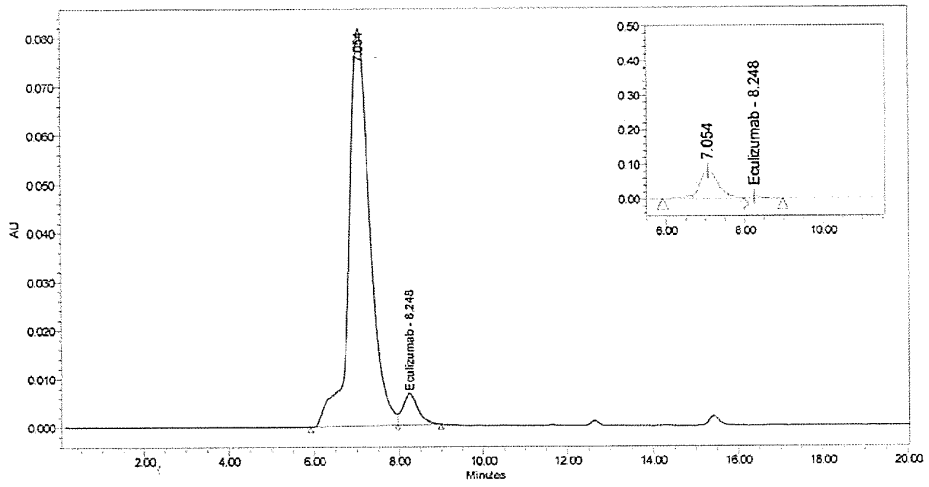
14 Jan 2019
Date

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Page 14208

Figure 1: Size Exclusion Chromatography of isolated dimer

SAMPLE INFORMATION

Sample Name:	Concentrated Dimer (26JUL05)	Acquired By:	tineg
Sample Type:	Unknow n	Date Acquired:	7/27/2005 11:47:18 AM
Vial:	2	Acq. Method Set:	Eculizumab SEC
Injection #:	1	Date Processed:	7/27/2005 1:53:16 PM
Injection Volume:	43.00 ul	Processing Method	Eculizumab SEC
Run Time:	20.0 Minutes	Channel Name:	Extract 214nm
Sample Set Name:	27JUL05GT01	Label:	



Peak Result Information

	Peak Name	RT(min)	Area (uV*sec)	Height (uV)	Int Type	% Area	Tailing @ 5%	Plate Count	USP Resolution
1		7.054	2795159	61657	BV	94.43	0.882	1179	
2	Eculizumab	8.248	164763	6579	VB	5.57		2358	1.513

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Page 14209

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ORAL PROCEEDINGS SCHEDULED FOR SEPTEMBER 5, 2019

September 3, 2019

Third Party Observations

Application Number	16 172 335.8
Publication Number	EP 3 124 029
Title	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT
Applicant	Alexion Pharmaceuticals, Inc.
Our file reference	AMG17203ADV-EP029

Third Party Observations

We would like to respectfully bring the following Third Party Observations to the Examining Division's attention:

The Applicant acted against the well-established principle of *nemo potest venire contra factum proprium*:

(1) In their submission dated August 28, 2018, Alexion attempt to defend their contradictive behavior in the proceedings regarding EP 1 720 571 and the current application. The Applicant's representative asserts that the principle of *nemo potest venire contra factum proprium* would not be applicable in this case (cf. p. 11, 4th par. of submission dated August 28, 2018). This is incorrect.

(2) The principle of the protection of legitimate expectations is a general principle well established in European Community law and generally recognized in the EPC contracting states and Board of Appeal case law (**G 2/97**, OJ 1999, 123; see also **R 4/09**). The term "good faith" is also used to describe this concept (**J 10/84**, OJ 1985, 71; **J 38/97**). Hence, it is established case law that users of the European patent system, who are

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PR-Nr. 1278

parties in proceedings before the EPO, must act in good faith (**G 2/97**, **R 4/09**).

(3) According to the "Saxe Declaration" submitted by the Applicant on August 28, 2018, Stephen Saxe submitted a letter to CAS dated April 28, 2009 explaining:

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

(4) Thus, Alexion were aware that they provided incorrect information regarding eculizumab to CAS at least as of April 28, 2009. Contrary to the Applicant's assertion, when filing the response of December 30, 2013 in the EP'571 case, Alexion thus did not make a *"mere assertion that turned out to be erroneous"* (cf. p. 11, 1st par. of submission dated August 28, 2018). Rather, they made an assertion and at the same time apparently assumed that the contrary was the case.

(5) In their submission dated July 5, 2019, Alexion argue that the copy of the CAS entry, provided with their letter in the opposition proceedings regarding EP 1 720 571 did not contain any sequences, that the identity of the sequences was not investigated, and that the argument was not challenged at that time (cf. p. 6, 2nd to 5th par. of letter dated July 5, 2019). We note that none of these allegations take away from the fact that Alexion have asserted before the OD in the EP'571 case that the sequence of eculizumab had been submitted to CAS knowing that this was incorrect.

(6) Alexion further try to defend themselves by arguing that they did not gain any advantage from their knowingly false statement (cf. p. 6, 6th par. of letter dated July 5, 2019). Of course, the obligation to act in good faith does not depend on the question whether a party gains an advantage or not.

(7) On the other hand, Alexion did not provide any evidence that they indeed filed the wrong structural information to CAS, since the Saxe Declaration - contrary to the assertion of Alexion's representative - does not include said "wrong" structure or any excerpt of the CAS registry showing that a "wrong" structure was submitted there. With the letter dated July 5, 2019, Alexion filed an excerpt from a CAS online resource allegedly showing the sequences originally submitted to CAS (**D28**). We note that the document provided by the Applicant does not include any information about the date on which the sequence shown therein has been submitted to CAS and/or been amended. This document, apparently retrieved on June 11, 2019, is therefore

unable to prove that the incorrect sequence has been submitted to CAS in 1999, i.e., twenty years ago.

(8) In this regard we would like to point out that the Applicant's allegation – that the patents and applications cited in **D8** would refer to anti-C5 antibodies more generally and that it would, therefore, be of no importance whether or not the correct sequence of eculizumab had been disclosed (p. 11, penultimate par. to p. 12, 1st par. of Applicants submission of August 28, 2018) - is simply not tenable. Rather, the question whether or not the correct sequence of the antibody designated via the INN eculizumab was available at CAS – published and provided with a CAS registry number - was of utmost importance.

(9) An INN is an **official** generic and non-proprietary **name** given to an active ingredient. The INN "eculizumab" is the **official** generic **name** given to the substance having the CAS-Number 219685-50-4. In conclusion, the above presented facts inevitably imply that the public – in particular authors of scientific literature but also the EPO - could rely upon the fact that a correct description of eculizumab was publicly available at the priority date.

(10) As will be explained below, during prosecution of EP 1 720 571 B1 and EP 2 359 834 B1, the Examining Division considered the term "eculizumab" at all times to disclose the specific antibody related to said INN. Alexion's representatives took the same position, even though Alexion apparently assumed at least as of April 2009 that the INN eculizumab related to a false structure (as they allege now).

(11) Claim 1 of EP 1 720 571 B1 as granted directly relates to eculizumab. It is readily derivable from the prosecution history (see first office action dated September 2, 2009 (enclosed as **Annex I**), Items 4.1 to 4.3 of the Communication; Summons to Oral Proceedings dated March 4, 2011 (enclosed as **Annex II**), Items 2.1, 5.1, 6.1) that the Examining Division heavily relied upon the fact that eculizumab was known on the priority date. In the Summons to Oral Proceedings the Examining Division further stated under Item 3.2 that:

> *"The application provides experimental details of dosage optimization of an anti-C5 antibody, 5G1.1* ***eculizumab****, for the treatment of PNH in a group of 11 patients.* ***Given the different affinities, functions and pharmacokinetics of individual, specific antibodies, the establishment of an optimal dosage schedule will be dependent on the antibody used****. Since independent claims 1-3 and 8-10 do not contain this feature it does not meet the requirement following from Article 84 EPC, taken in combination with Rule 43(1) and (3) EPC, that any independent claim must contain all the technical features essential to the definition of the invention."*

(12) Although Alexion assumed that eculizumab was not disclosed at the priority date, as they now allege, the disclosure of eculizumab was confirmed by them vis-à-vis the Examining Division (see reply to Summons to Oral Proceedings dated September 23, 2011 (enclosed as **Annex III**), page 9, second paragraph). Because the application as filed did not disclose the actual structure of eculizumab this was the only way to overcome the cited objection.

(13) The sole claim of EP 2 359 834 B1 as granted directly relates to eculizumab. Again, the Examining Division heavily relied upon the notion that eculizumab had been disclosed before the priority date (see e.g. European Search Opinion dated July 21, 2011 (enclosed as **Annex IV**), objection under Item 2, upheld by the Examining Division in further prosecution). The prior disclosure of eculizumab was also confirmed by Alexion *vis-à-vis* the Examining Division (see reply to Office Action dated November 15, 2012 (enclosed as **Annex V**), Item 2.2.2):

> *"This understanding is further underlined by the general knowledge in the art of antibody engineering and the skilled person's understanding of the structural features of eculizumab, for example, which was known to comprise "a G2/G4 [constant region] in place of the G1 domain" (see specification at page 5, lines 16 to 18; see also the package insert for Soliris®, the trade name under which eculizumab is sold, at paragraph 11 (a copy of the package insert is submitted herewith as Exhibit A)). Applicant also provides Mueller et al. (1997) Mol Immunol 34(6):441-452 as Exhibit B, which describes a G2/G4 Fc portion of an antibody as lacking complement activating activity (see, e.g., page 446, column 2 and Fig. 9)."* (Emphasis added.)

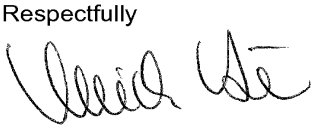
(14) Again, Alexion relied upon the public's knowledge of the structure of eculizumab before the Examining Division to support the patentability of claims to "eculizumab" but now assert that one of skill in the art lacked such knowledge.

(15) In summary, the WHO named eculizumab to be the official generic name given to the substance having the CAS-Number 219685-50-4. According to their own statements, Alexion were aware of having provided incorrect information regarding eculizumab to CAS at least as of April 28, 2009. Therefore, the concealment of this information cannot be considered to merely have been erroneous. By concealing this information Alexion cannot be said to have acted in good faith when attempting to overcome the various objections from the Examining Division, which objections could not have been overcome had the EPO been informed that the INN eculizumab was in fact assigned to a false structure. On the other hand, there is no evidence provided that Alexion have indeed filed "wrong" structural information to CAS. At the moment, this appears to be a mere assertion.

(16) In conclusion, Alexion's conduct vis-à-vis the EPO is contrary to the principle of *nemo potest venire contra factum proprium*, and their attempt to justify their concealment of alleged facts, which, if true would have been critical for validity in a number of proceedings, and which are

nonetheless now supposed to serve as basis to restore novelty over prior art documents, must be refused.

Respectfully



Dr. H. Ulrich Dörries

Enclosures
Annexes I-V

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Our ref: SK/6929 Your ref: **EP 3 124 029** Wroclaw, September 4th, 2019

Re: **Third Party Observations in respect of EP 3 124 029**

THIRD PARTY OBSERVATIONS

European Patent:	Application Number: EP16172335.8
	Publication Number: EP 3 124 029
Applicant:	Alexion Pharmaceuticals, Inc.
Title:	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

Dear Sirs,

We enclose the Third Party Observations in respect of EP 3 124 029 for attention of Examining Division.

Your faithfully

Rafał Witek
Patent Attorney

Enclosures:

1. Third Party Observations in respect of EP 3 124 029
2. EP1558648_Request for correction
3. EP1558648_Original filing (WO 2004/035607 A2)
4. EP1558648_Vink declaration
5. EP2581113_Request for correction
6. EP2581113_Application as filed

4th September, 2019

EP Appl. No. 16 172 335.8 (EP 3 124 029)
In the name of Alexion Pharmaceuticals, Inc.

Third Party Observations

We would like to respectfully bring the following Third Party Observations to the Examining Division's attention:

I. The amended claims submitted by the Applicant as Main Request contravene Articles 123(2) and 76(1) EPC

(1) Throughout Examination proceedings, the Applicant has argued that the skilled person would have immediately recognized that the Applicant could not have intended to seek protection for an antibody comprising all of SEQ ID NO: 3 or all of SEQ ID NO: 4 because he/she would immediately have seen that these sequences contain a signal peptide (leader sequence) and would know that it is not possible to make a mature antibody that contains a signal peptide.

(2) The Applicant pointed to Enlarged Board of Appeal decisions **G 3/89** and **G 11/91** to support his allegations. We note that the Applicant's arguments are based on a flawed interpretation of the cited case law.

(3) A correction under Rule 139 EPC is of a strictly declaratory nature. Two requirements arise for a correction under Rule 139 to be allowable, i.e., not to contravene Article 123(2) EPC and/or Article 76(1) EPC. First, the presence of an error must be obvious. Second, it has to be *"immediately evident [for a skilled person] that nothing else would have been intended than what is offered as the correction"* (Rule 139 EPC, second sentence). If there is any doubt, including even slight ambivalence, that nothing else would have been intended than what is offered as the correction (i.e., the correct sequence of eculizumab), a correction cannot be allowed (see also **J 16/13**, points 8 to 10 of the reasons).

(4) The Proprietor has not met these requirements as they have not shown that it would be immediately evident that the leader sequence of SEQ ID NOs: 3 and 4 would precisely consist of residues 1-22 of SEQ ID NOs: 3 and 4 and nothing else.

(5) The Proprietor alleges in this regard (p. 3, 5th par. of the submission dated August 28, 2018) that the skilled person would know from his common general knowledge that the *"framework of human kappa light chains commonly starts with the residues Asp Ile Gln Met as shown in the Table at pages 103 to 108 of Kabat et al."* (Exhibit E filed with the Andrien Declaration on May 31, 2017). Notably, the table of contents (Exhibit C filed with the Andrien Declaration on May 31, 2017) makes clear that the Applicant

chose to submit only a fraction of the human kappa light chain sequences disclosed in Kabat *et al.* (according to the table of contents, human kappa light chain sequences are disclosed on pages 103 to 130 of Kabat *et al.* out of which only pages 103 to 108 have been submitted). The sequence variability among human kappa light chains must therefore be assumed to be considerably greater than what is derivable from the documents submitted by the Applicant.

(6) However, even within the limited selection presented by the Applicant, Asp lle Gln Met are at best commonly the first residues. When assessing the allowability of an amendment under Rule 139 EPC, the question is not whether human kappa light chains commonly start with any particular sequence, but whether they must start with a certain sequence and whether this would have been obvious to the skilled person. Otherwise, the skilled person would be left with uncertainty, at which specific residue of SEQ IDs NO: 3 and 4 the correct mature sequence (excluding the leader peptide) would begin. This is clearly the case here, which will we explained in more detail below.

(7) Specifically, one question is how many residues the skilled person would have subtracted from the sequence disclosed in the patent, if recognizing that said sequences comprise a leader peptide? Further, how would the skilled person arrive at one and only one possible sequence to deduct from SEQ ID NOs: 3 and 4? The Proprietor states that *"the last two residues [of the leader peptides] are predominantly Arg Cys"* (par. bridging p. 2/3 of the submission). Again, as the Examining Division will be aware, *"predominantly"* does not meet the standard required for a correction under Rule 139 EPC. Quite to the contrary, the fact that the Applicant uses the term "predominantly" already shows that there are alternatives to the correction that would be apparent to the skilled reader of the application. However, as noted above, there must not be any ambiguity for a correction to be allowable. Otherwise, the skilled person would be confronted by the correction with subject matter that is not originally disclosed.

(8) The Applicant thus failed to show that a skilled artisan would immediately recognize how to replace the incorrect sequences (i.e., what the specific sequence of the leader peptide would be), with certainty.

(9) The Applicant's representative further asserted that similar requests for corrections would have been accepted by the EPO, pointing to EP 1 558 648 and EP 2 581 113 (cf. submission dated August 28, 2019, p. 4, penultimate par.). Regarding EP 1 558 648, the relevant protein sequences as well as the CDR sequences were derived by standard sequencing procedures (Example 2, WO 2004/035607 A2, submitted herewith). The antibodies were then claimed by reference to V_L and V_H sequences or to CDR sequences which is also standard in the art. Moreover, the original application disclosed at p. 29, I. 35-36 that *"heavy and light chain leader sequences are cleaved during protein maturation and do not contribute to the properties of the final antibody"*.

(10) The Applicant of EP 2 581 113 had pointed to page 67, paragraphs [0222] and [0223] of the application as filed (submitted herewith), disclosing that the presence of a signal sequence in a VL or VH amino acid

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sequence can be determined by comparing the length of the VL or VH amino acid sequence with those of known antibodies.

(11) Both applications referred to by the Applicant therefore discuss the occurrence of leader sequences or a clear identification of the mature antibody by comparison to known peptides. As pointed out above, the current application as filed is completely silent about leader sequences and does not provide any guidance for the skilled person how to clearly identify the claimed antibody. Therefore, these cases cannot be compared to the present one.

(12) Notably, with the request for correction of an obvious error, the Applicant in the EP 1 558 648 case submitted an expert declaration of Tom Vink (attached hereto). In the declaration, the expert explains the following (section 10 of the Vink declaration):

> 10. The leader sequence for a V_H sequence is typically 19-21 amino acids long, and the leader sequence for a V_L kappa sequence is typically 19-23 amino acids long. A protein always starts with a Met residue, so the leader sequences always start with a Met residue. A V_H sequence may start with a Glu or Gln residue, a V_L kappa sequence may start with Ala, Asp, Val, Asn or Glu. V_H signal peptides can end with Cys, Ala, Ser or Pro, and V_L kappa signal peptides can end with Cys, Ala, Gly or Glu. Accordingly, a person skilled in the art would know from studying the nucleotide and amino acid sequences (SEQ ID Nos: 1-12) that the leader sequences are indeed included. Also a person skilled in the art would note that the amino acid sequences are longer than usual for the mature amino acid sequences. This information was known to a person skilled in the art at the earliest priority date of the application and further supports that the leader sequences are indeed included in the variable heavy chain and light chain amino acid sequences as identified by SEQ ID Nos: 2, 6, 10, 4, 8 and 12, respectively.

(13) According to the statement of the expert, V_L kappa signal peptides typically have a length of 19 to 23 AAs and end with C, A, G or E, while human V_L kappa chains begin with A, D, V, N or E. We note that the number of possible combinations appears to be even larger, when taking Kabat *et al.* into regard (see above). However, if this reasoning is applied to the sequences disclosed in EP 3 124 029 as filed originally (see below) at least one alternative cleavage site to the one suggested by Alexion would be apparent to the skilled person, namely between amino acids 19 and 20 (WLR**GA**RC). The resulting leader peptide would be 19 amino acids in length, end with a G residue and the resulting mature V_L chain would begin with an A residue. Thus, all requirements that the skilled person would allegedly be aware of (according to the Vink declaration) would be fulfilled.

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SEQ ID NO: 3 – Eculizumab V_L

MDMRVPAQLLGLLLLWLRGARCDIQMTQSPSSLSASVGDRVTITCGASENIYGAL
NWYQQKPGKAPKLLIYGATNLADGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQ
NVLNTPLTFGQGTKVEIKRT

SEQ ID NO: 4 – Eculizumab Light chain

MDMRVPAQLLGLLLLWLRGARCDIQMTQSPSSLSASVGDRVTITCGASENIYGAL
NWYQQKPGKAPKLLIYGATNLADGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQ
NVLNTPLTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKV
QWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG
LSSPVTKSFNRGEC

(14) This is clear evidence that the skilled artisan would in the present case at the very least be confronted with two alternatives, when recognizing that SEQ IDs NO: 3 and 4 of the current application as filed erroneously comprise a leader peptide. Under such circumstances, the correction under Rule 139 EPC suggested by the Applicant would inevitably add information that is not originally disclosed.

(15) We lastly note that the *Kabat et al.* reference, as submitted by the Applicant, represents only a small (and potentially purposeful) selection from an amino acid sequence compendium that itself represents only a selection from the vast space of protein, or antibody sequences. It cannot provide evidence that nothing else than the sequences disclosed therein would have been thinkable sequences of signal peptides, or mature human kappa light chains for the skilled person.

(16) This is aggravated by the fact that the 5th edition of Kabat *et al.* was published in 1991, i.e., 15 years prior to the priority date of the application. In the quickly moving field of pharmacologically active antibodies, the space of known antibody sequences has significantly expanded within this period and thus, the sequence compendium referred to by the Applicant was long outdated at the priority date. The number of mature light chain sequences known in the art was certainly much larger in 2006, compared to 1991, accordingly, the number of possible options for the length and/or sequences of signal peptides was likely much larger in 2006, compared to 1991. Thus, the skilled person would have been confronted with even more uncertainty regarding the sequence and length of the signal peptide comprised in SEQ ID NOs: 3 and 4, even if recognizing that the sequences disclosed therein are immature.

(17) Thus, Kabat *et al.* is anyway not representative of the entire space of possible sequences the skilled person would have had to consider at the relevant date.

(18) In summary, the application provides no suggestion that specifically residues 1-22 of SEQ IDs NO: 3 and 4 are necessarily leader sequences, let alone that nothing else would have been intended than what the Applicant offered as correction. It can by no means be assumed that it would have been apparent to the skilled person that the sequence submitted by the Applicant is the only conceivable replacement for the sequences originally disclosed.

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(19) Therefore, the Applicant's request for correction of the description and the reference to residues 23 to 214 of SEQ ID NO:4, introduced into claim 1 of the MR contravene Articles 123(2) and 76(1) EPC.

II. The Auxiliary Requests filed on July 5, 2019

(20) With the submission dated July 5, 2019, the Applicant has submitted two Auxiliary Requests, AR1 and AR2. The Auxiliary Requests not only fail to overcome the objections raised by the Examining Division, at least AR1 introduces further violations of the EPC. This is explained in further detail below.

II.1 Auxiliary Request 1

(21) AR1 defines the claimed antibody by reference to SEQ ID NOs 2 and 4, however, the claim language has been amended such that claim 1 relates to an *"antibody that binds C5 [...], wherein the antibody comprises the heavy chain amino acid sequence shown in SEQ ID NO: 2 and the light chain amino acid sequence shown in SEQ ID NO: 4"* instead of *"comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4"*.

(22) This alternative claim language is a clear violation of Articles 76(1) and 123(2) EPC. The application as filed refers to an antibody comprising a heavy chain and a light chain consisting of SEQ ID NOs: 2 and 4, respectively (cf. application as filed at p. 5, li. 30 to 33). In contrast, claim 1 of AR1 refers to an antibody which comprises the heavy and light chain sequences "shown in" SEQ ID NOs 2 and 4. The Applicant thus effectively replaced "consisting of" by "comprising", which in itself represents a violation of Articles 76(1) and 123(2) EPC, as the terms are not freely interchangeable (**T 759/10**).

(23) Thus, while the application as filed defines the antibody in a closed manner (heavy and light chains consisting of SEQ ID NOs 2 and 4), claim 1 of AR1 encompasses antibodies that comprise the sequences "shown in" SEQ ID NOs 2 and 4, but may, due to the open claim language, also comprise further sequence elements. For example, an antibody comprising a heavy and a light chain, wherein the heavy chain comprises the amino acid sequence of SEQ ID NO: 2 elongated by a few further amino acids at the C-terminus would fall under claim 1 of AR1. The original disclosure does not teach such modified antibodies.

(24) The alleged basis provided by the Applicant in the letter dated July 5, 2019 (original application, p. 4, li. 32 and p. 44) fails to disclose the subject matter of claim 1 of AR1. The description text of the parent application as filed and of the divisional application as filed are identically worded. Page 4, line 32 of the original application teaches that *"[i]n certain embodiments, said antibody is eculizumab"* (emphasis added). This passage neither provides basis for the "comprising" language, nor for the vague "shown in"

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phrase of claim 1 of AR1. Page 44 of the application discloses the (partially incorrect) SEQ IDs. Taken together, these two passages indicate to the skilled person that the antibody disclosed in the application may be an antibody named "eculizumab", which consists of heavy chains and light chains, wherein the heavy and light chains consist of the sequences disclosed as SEQ ID NOs 2 and 4. A widening of this concept through the use of "comprising" language cannot be based on this disclosure.

(25) In addition, the introduction of the term "shown in" leads to a further violation of Articles 76(1) and 123(2) EPC. This is because different interpretations of this phrase are possible, at least some of which would introduce subject matter that lacks original disclosure. If *"the light chain amino acid sequence shown in SEQ ID NO: 4"* means "the light chain amino acid sequence of SEQ ID NO: 4", claim 1 of AR1 would refer to an antibody that is not eculizumab, and thus, an antibody for which protection is not actually sought, as explicitly stated by the Applicant (e.g., letter of July 5, 2019, p. 15, 1st full par.). The amendment would furthermore not solve the objections of the ED with regard to insufficiency of disclosure.

(26) Alternatively, the term "shown in" could be interpreted as meaning any light chain sequence(s) comprised in SEQ ID NO: 4. It may be assumed that this is the interpretation desired by the Applicant, because this would enable him to *inter alia* cover the (correct) light chain sequence of eculizumab via reference to the (incorrect) sequence disclosed in the application text (SEQ ID NO: 4). However, in this case, too, the amendment would not solve the objections of the ED with regard to insufficiency of disclosure, since a light chain amino acid sequence of SEQ ID NO: 4 would still be encompassed. Further, since SEQ ID NO: 4 encompasses at the very least two potential light chain sequences when interpreted in light of common general knowledge (cf. above), this claim interpretation would inevitably lead to the finding that claim 1 adds matter, since it now encompasses several (potential) light chain sequences, instead of one clearly defined light chain sequence.

(27) The introduction of the vague term "shown in" furthermore renders claim 1 wholly unclear. As explained above, it is not clear to the skilled person which exact sequence(s) are encompassed by claim 1 of AR1. The heavy and light chain sequences of SEQ ID NOs 2 and 4, or any heavy and light chain sequence(s) that are part of SEQ ID NOs 2 and 4, i.e., any sequence fragment that can be extracted from SEQ ID NOs 2 and 4 and that may serve as an antibody heavy, or light chain, respectively - together with further derivatives thereof comprising additional amino acids?

(28) In summary, AR1 violates at least Articles 84, 76(1) and 123(2) EPC.

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II.2 Auxiliary Request 2

(29) Claim 1 of AR2 defines the claimed antibody by reference to *inter alia "residues 23 to 214 of SEQ ID NO: 4"*. As explained above, this reference to only part of SEQ ID NO: 4 is not derivable from the application as filed. Claim 1 of AR2 therefore suffers from the same deficiencies under Article 76(1) and 123(2) EPC as claim 1 of the Main Request.

Encl.
EP1558648_Request for correction
EP1558648_Original filing (WO 2004/035607 A2)
EP1558648_Vink declaration
EP2581113_Request for correction
EP2581113_Application as filed

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J A • K E M P

BY ONLINE FILING

The European Patent Office
Bayerstrasse 34
(entrance via Zollstrasse 3)
80335 Munich
Germany

5 July 2019

Dear Sirs

European Patent Application No. 16195355.9
ALEXION PHARMACEUTICALS, INC.
Our Ref: N411599EP-B PXT

Thank you for the summons to attend oral proceedings dated 27 March 2019. The Applicant wishes to make the following submissions in advance of the hearing scheduled for 5 September 2019.

The claims currently on file form the basis of our main request.

Three auxiliary requests are attached and we ask that the Examining Division consider the auxiliary requests in the event that the main request is considered unallowable.

Tracked-changes versions of the main request showing the amendments being made to derive the first two auxiliary requests and of the first auxiliary request showing the amendments being made to derive the third auxiliary request are attached.

I attach a full list of the documents cited in these examination proceedings. A copy of each of D24 to D31 is attached.

The present claims

Claim 1 of the present application is directed to:

> "*An antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4.*"

Claim 2 is directed to a pharmaceutical composition comprising this antibody.

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The present application discloses that SEQ ID NO: 2 is the full (variable and constant) heavy chain amino acid sequence of eculizumab, and that SEQ ID NO: 4 is the full (variable and constant) light chain amino acid sequence of eculizumab. This is confirmed in paragraph 6 of the Boone declaration (D13). SEQ ID NO: 2 is the mature sequence and does not include the N-terminal signal peptide. The heavy chain constant region of SEQ ID NO: 2 is a unique, engineered heavy chain constant region. SEQ ID NO: 4 is an immature sequence that includes the 22 amino acid N-terminal signal peptide, which is cleaved off during antibody production in a cell. The light chain constant region of SEQ ID NO: 4 is a kappa constant region.

The claims are therefore directed to the specific antibody, eculizumab, which obtained marketing approval under the trade name SOLIRIS® after the filing date of the present application (see D15/D16).

The structure of eculizumab was not disclosed in the prior art

The full heavy chain and light chain amino acid sequences of SOLIRIS® (eculizumab) were first disclosed in the present application. Prior to the filing date of the present application, it was not known that "eculizumab" was an antibody comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4.

D1-D5 do not disclose the structure of the claimed antibody

None of D1-D5 includes any structural information for "eculizumab".

D1 refers to "eculizumab" as being the tradename of the antibody "*h5G1.1-mAb*". D1 does not include any sequence information, but states that:

> "*Methods for the preparation of h5G1.1-mAb, h5Gl.l-scFv and other functional fragments of h5G1.1 are described in U.S. Pat. No. 6,355,245 and "Inhibition of Complement Activity by Humanized Anti-C5 Antibody and Single Chain Fv", Thomas et al., Molecular Immunology, Vol. 33, No. 17/18, pages 1389-1401,1996, the disclosures of which are incorporated herein in their entirety by this reference. The antibody h5G1.1-mAb is currently undergoing clinical trials under the tradename eculizumab."* (D1, page 5, left column last two lines to right column line 8).

U.S. Pat. No. 6,355,245 is D11, and Thomas et al., Molecular Immunology, Vol. 33, No. 17/18, pages 1389-1401,1996 is D7 in the present proceedings.

D2 describes "eculizumab" as "*a humanized monoclonal antibody that binds C5 and inhibits terminal complement activation*" (D2, lines 4-5). No structural information is provided in D2, and it does not reference any other documents.

D3 describes "eculizumab" as "*a recombinant humanized monoclonal antibody that was designed to block the activation of terminal complement components*[14,15]" (D3, left column page 553, third paragraph, first three lines). Reference 14 in D3 is a general publication relating to humanization of antibodies (Riechmann et al. (1988) "Reshaping human antibodies for therapy" Nature 332: 323-7), and reference 15 is D7 in the present proceedings. No structural information is provided in D3 itself.

D4 describes "eculizumab" as "*a recombinant, fully humanized monoclonal antibody that inhibits the activation of terminal complement components. Eculizumab binds specifically to the terminal complement protein C5 and prevents the release of the inflammatory mediator C5a and the formation of C5b-9 by inhibiting the cleavage of human complement C5 to C5a and C5b (12)*" (D4, page 673, right column, third paragraph, first three lines). Reference 12 in D4 is D7 in the present proceedings. D4 also includes the CAS STN database number "CAS: 219685-50-4" on page 673. However, as discussed below, this database did not include the correct sequence for eculizumab at the filing date of the present application. No structural information is provided in D4 itself.

D5 defines "eculizumab" as "*a monoclonal antibody that blocks the complement system at C5*" (D5, page 849, left column, last two lines of answer to first question). No structural information for "eculizumab" is provided in D5.

Thus, D1 to D5 do not provide any sequences for "eculizumab", and only point to D7 and/or D11 as describing "eculizumab".

Neither D7 nor D11 discloses the full structure of the claimed antibody

D11 and D7 are referred to in the cited prior art documents as describing "eculizumab". However, neither D7 nor D11 discloses the amino acid sequences of SOLIRIS® (eculizumab) recited in the present claims.

D7 and D11 describe the cloning of the murine 5G1.1 antibody and the generation of humanised variable region sequences.

The only disclosure relating to full length antibodies in D11 is a general paragraph at column 45, lines 24-33 which describes in general terms how matched pairs of the variable regions (e.g., a VL and a VH region) of the various described antibody molecules, Fds and light chains may be combined with constant region domains to form full length antibodies of the invention.

D7 discloses CDR grafted V_L and V_H sequences (h5G1.1VCH and h5G1.1VLC in Figure 1 of D7) and also V_L and V_H sequences comprising back mutations (h5G1.1VHC+F and h5G1.1VLC+F in Figure 1 of D7) and reports that an intact humanised antibody of the IgG4 isotype was constructed and produced (see page 1396, paragraph spanning columns of D7).

As discussed above, the present claims are directed to an antibody comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4, which are the sequences of the antibody in SOLIRIS® (eculizumab). This claimed antibody is **not** the IgG4 antibody described in D7. In particular, eculizumab does not have an IgG4 heavy chain constant region, but a unique, engineered, non-naturally occurring constant region sequence that has 14 different amino acid residues compared to the IgG4 sequence (4 in the CH1 domain, 6 in the hinge region and 4 in the CH2 domain - see paragraphs 4 and 5 of the declaration of Dr Leonard Bell (D12)).

Therefore, although D1 refers to D7 and D11, and D3 and D4 refer to D7, supposedly as describing "eculizumab", neither D7 nor D11 discloses the full heavy chain sequence shown in SEQ ID NO: 2 of the present application and the full light chain sequence shown in SEQ ID NO: 4 of the present application.

With regard to D11, the Examining Division has argued that:

> "*The description of the invention (see page 16, lines 19-24) refers to D11 and states that the document discloses the preferred whole antibody named eculizumab. That would mean that the eculizumab claimed in the patent and the one disclosed in D11 is the same antibody*" (page 7, first paragraph of the summons dated 23 March 2019).

Page 16, lines 24-25 of the present application states that: "*These antibodies disclosed in U.S. Patent 6,355,245 [D11] include a preferred whole antibody (now named eculizumab)*". However, it is necessary to look at D11 to see what it actually discloses, and despite the statement made in the present application, D11 does not in fact disclose the sequences of any whole antibody.

As discussed above, the only disclosure relating to full length antibodies in D11 is a general paragraph at column 45, lines 24-33 which describes in general terms how matched pairs of the variable regions (e.g., a VL and a VH region) of the various described antibody molecules, Fds and light chains may be combined with constant region domains to form full length antibodies of the invention.

The statement in the present application is therefore correctly interpreted as meaning that D11 discloses a genus of antibodies that includes the preferred antibody of the present invention (eculizumab), and not that this antibody is explicitly disclosed in D11. It is a matter of fact that D11 does not disclose an antibody with a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4 as presently claimed.

None of D1-D5, D7 and D11 provide an enabling disclosure of the claimed antibody

In view of the above, the skilled person would not be able to reproduce "eculizumab" based on the disclosures of any of D1-D5, D7 and D11. According to the established case law, a disclosure in a prior art document is novelty-destroying only if the teaching it contains is reproducible (see, for example, T 1437/07, Reason 25, and Guidelines for Examination G-IV-2).

Hence, none of D1-D5 can be considered to belong to the state of the art according to Article 54(2) EPC, at least in as far as they relate to "eculizumab", and consequently none of D1-D5 prejudices the patentability of the claimed invention.

None of the patents listed in D8 provide an enabling disclosure of the claimed antibody

D8 lists a number of Alexion patents and patent applications that refer to "eculizumab". None of the patents and patent applications listed in D8 provide any sequence information for "eculizumab". Insofar as these patents and patent

applications use the term "eculizumab", they refer back to D7 and/or D11 as shown in the attached table (D24).

As discussed above, neither D7 nor D11 disclose an anti-C5 antibody as presently claimed, with a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4. These full sequences of the antibody in SOLIRIS® (eculizumab) were not known in the art at the filing date of the present application. Therefore, the prior art disclosures in the patents and patent applications do not enable the skilled person to make the specific antibody that is now claimed.

In this regard, it should be noted that the patent and patent applications listed in D8 are not focused only on eculizumab. These patents and applications relate to complement inhibitors more broadly, including anti-C5 antibodies generally. As shown in D8, with one exception (EP 1720571, the patent granted from D10, which has been revoked (see below)), "eculizumab" is referred to only in dependent claims. Therefore, the fact that there is no enabling disclosure of the specific antibody "eculizumab" does not render all of these patents and patent applications insufficient across their whole scope. The insufficiency extends only to the specific dependent claims that mention "eculizumab."

Comments made by Alexion in D9 in the course of opposition proceedings regarding a different patent do not change what was in fact disclosed in the prior art

The Examining Division considers that D10 destroys the novelty of the present claims because the applicant stated in D9 that the structure of the antibody had been made available to the public on 14 February 1999. However, the statement made in 2013 by the applicant in the opposition proceedings against the patent derived from D10 does not change what was disclosed in D10 in 2005, and does not retroactively make the disclosure of D10, or any other prior art document, enabling in so far as it relates to "eculizumab".

The issue of sufficiency of disclosure of "eculizumab" was raised in the opposition proceedings against EP 1720571 (the patent derived from D10). The Opponent observed that:

> *"Eculizumab is an essential feature of claims 1 to 8. The structure of Eculizumab is not disclosed in the application as filed. On page 8, lines 8-9, the patent discloses that h5G1.1-mAb is currently undergoing clinical trials under the tradename Eculizumab. On the same page, lines 5 to 8, the patent further mentions that methods for the preparation of h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1 are described in U.S. Patent No. 6,355,245 (D9)* [D11 in the present proceedings] *and 'Inhibition of Complement Activity by Humanized Anti-C5 Antibody and Single Chain Fv,' Thomas et al., Molecular Immunology, Vol. 33, No. 17/18, pages 1389-1401, 1996 (D10)* [D7 in the present proceedings].
>
> ***However, as detailed below, the complete sequence of Eculizumab or h5G1.1 mAb is not disclosed in these two documents.***

> *D9* [D11] *is a patent assigned to Alexion Pharmaceuticals, Inc. which relates to C-5 specific antibodies for the treatment of inflammatory diseases.*
>
> *In example 7, D9* [D11] *discloses a mouse monoclonal antibody 5G1.1. In example 11 (columns 42 to 44), D9* [D11] *lists a number of humanized, chimeric and non humanized ScFv, Fd and light chains. D9* [D11] *does not comprise any reference to Eculizumab or h5G1.1 mAb nor does it disclose the sequence of a full length humanized antibody.*
>
> *D10* [D7] *is a publication from Alexion Pharmaceuticals relating to humanized anti-C5 antibody and scFv. D10* [D7] *discloses the cloning of the variable region genes of the mouse mAb 5G1.1 (see page 1380), the humanization of 5G1.1 variable regions and the construction and expression of various Fab and scFv (see page 1391). The sequences of humanized variable regions are provided in Figure 1. The construction of a humanized antibody 5G1.1 called h5G1.1 HuG4 is also discussed on page 1396 (paragraph bridging col. 1 and 2) and on page 1399, col.2. However, the exact sequence of the humanized antibody is not disclosed in D10* [D7].
>
> *In view of the above, none of the patent, D9* [D11] *or D10* [D7] *provides a detailed description of the structure of h5G1.1 mAb or Eculizumab.*
>
> *Therefore, the man skilled in the art would not be able to carry out the invention as he would not be able to prepare the specific antibody Eculizumab, which is an essential feature of the claims 1 to 8."* (See section 7.1 of D21)

In defence of its patent, Alexion replied to this attack by asserting that the sequence for eculizumab was publicly available and stated (in D9) that:

> "A *sequence for eculizumab was submitted to Chemical Abstracts Service (CAS) and entered into their STN database on 14 February 1999; please see the attached copy of the CAS entry no. 219685-560-4."*

The copy of CAS entry no. 219685-560-4 provided did not include any sequences, the identity of the sequences made available on 14 February 1999 was not investigated at that time, and this argument was not challenged.

In the preliminary opinion that accompanied the summons to oral proceedings dated 14 January 2014 (D25), the Opposition Division expressed the opinion that:

> "I*n the absence of evidence that* eculizumab *could be identified as a specific antibody, either by deposit or* ***full*** *sequence of its light and heavy chains, the antibody and thus the subject-matter of claims 1-8 cannot be considered to be sufficiently disclosed" (paragraph 2.29 of D25).*

The position of the Opposition Division was not further challenged by Alexion or discussed at the oral proceedings (see Alexion letter of 17 March 2014 (D26), which does not include any arguments relating to sufficiency of disclosure), and EP1720571 was revoked for lack of inventive step (see the Decision of the Opposition Division dated 24 June 2014 (D27)).

The statement made in defence of EP1720571 should have no bearing on the current proceedings. It does not change the facts of what was disclosed prior to the filing date of the present application. Nor did Alexion gain any advantage from the statement made in connection with the opposition to that earlier patent.

In accordance with the opinion of the Opponent and Opposition Division in the opposition against EP1720571 that this patent does not provide a sufficient disclosure of "eculizumab", the application for EP1720571, D10, also does not provide an enabling disclosure of "eculizumab" and does not destroy the novelty of the present claims, which are directed to the unique, engineered antibody in SOLIRIS®.

The sequence of eculizumab was not available in the CAS database at the filing date

Heavy and light chain amino acid sequences were submitted to the CAS Registry in 1999 under the name "eculizumab", but, as evidenced by the Saxe Declaration, the sequences submitted to CAS at this time were **not** the sequences of the antibody (eculizumab) in SOLIRIS® to which the present claims are directed. The correct sequences for eculizumab were later submitted to replace the incorrect sequences in May 2009. These facts are not disputed.

However, one argument put forward by the Examining Division is that the availability of incorrect sequences for eculizumab on the CAS Registry database amounts to a public disclosure of the correct sequences for eculizumab because it was the intention of Alexion to make the sequence of eculizumab public at the time, such that Alexion would be obliged to provide the correct sequence of eculizumab to anybody who requested it. The Examining Division has referred to "*CAS rules*" as obliging Alexion to provide such information. However, the Examining Division has not provided evidence that any such rules existed, and Alexion is not aware of any. The fact therefore remains that the correct sequence of eculizumab was not made available in the CAS Registry prior to the filing date of the present application.

The statement by the applicant in D9 ("A *sequence for eculizumab was submitted to Chemical Abstracts Service (CAS) and entered into their STN database on 14 February 1999; please see the attached copy of the CAS entry no. 219685-560-4.")* does not change the fact that the wrong sequence was included in the CAS database in February 1999 and the fact that this incorrect sequence was still the sequence listed for "eculizumab" in the CAS database at the filing date of the present application. The context of the statement in D9 is explained above. Rather than speculate as to the intent of Alexion in 1999, the proper inquiry should be what in fact was disclosed at the filing date of the present application and whether a person skilled in the art would have known or been able to determine the structure of eculizumab, as recited in the present claims.

The Boards of Appeal have considered the prior art effect of documents that contain mistakes. In particular, as stated in the Case Law of the Boards of Appeal, 8th edition, I-C-4.9, in T 412/91 the board held that:

> *"in principle, what constituted the disclosure of a prior art document was governed not merely by the words actually used in its disclosure, but also by*

> *what the publication revealed to the skilled person as a matter of technical reality. If a statement was plainly wrong, whether because of its inherent improbability or because other material showed that it was wrong, then - although published - it did not form part of the state of the art. Conversely, if the skilled person could not see the statement was wrong, then it did form part of the prior art.*"

Similarly, in the Case Law of the Boards of Appeal, 8th edition, I-C-4.9, it is noted that:

> "*In T 230/01 the board noted that a document normally forms part of the state of the art, even if its disclosure is deficient, unless it can unequivocally be proven that the disclosure of the document is not enabling, or that the literal disclosure of the document is manifestly erroneous and does not represent the intended technical reality. Such a non-enabling or erroneous disclosure should then not be considered part of the state of the art.*"

Applying this reasoning to the present case, either the skilled person would recognise that the sequences of eculizumab in the CAS database must be wrong, in which case the incorrect information is not part of the state of the art, or the sequences in the CAS database do not destroy the novelty of the present claims.

The Board in T 591/90 considered that it could be assumed that a skilled person would try to correct recognisable errors in a prior document if, using his general technical knowledge and consulting the reference literature, he could see at once that the information in question was not correct. (See Case Law of the Boards of Appeal, 8th edition, I-C-4.9.)

In the present case, the skilled person would be unable to correct the errors in the eculizumab sequences in the CAS database based on his common general knowledge and by consulting the literature.

The Saxe declaration (D17) explains that the heavy chain sequence submitted to CAS was much longer (650 amino acids) than the actual heavy chain sequence (448 amino acids). The annotated copy of the sequences originally submitted to CAS attached as D28 (obtained by searching for CAS Registry Number: 219685-50-4 on http://commonchemistry.org/ and annotated by hand) shows how extensive the errors are. In particular, the heavy chain sequence originally submitted to CAS contains three stretches of additional amino acids, has two single amino acid substitutions and is missing a stretch of 16 amino acids compared to the correct sequence. For comparison, an annotated copy of the corrected sequences for CAS Registry Number: 219685-50-4 is attached as D28a.

As discussed above, the skilled person would not have known that eculizumab has a uniquely-engineered heavy chain, and so the skilled person would not have known what the correct heavy chain constant region sequence of eculizumab should be.

Also, the light chain submitted to CAS in 1999 contains a single amino acid substitution compared to the correct sequence in the variable region (see D28 and D28a). The light chain variable region sequence shown in D7 is the same as the

incorrect sequence at this position, and so the skilled person would have had no way of knowing that there was an error in the light chain variable region sequence.

Accordingly, the errors in the eculizumab sequences are not errors that would have been recognisable, or at least correctable, by a person skilled in the art at the filing date.

It is worth noting how the EPO would have treated an earlier patent application in which the Applicant had erroneously included the incorrect sequence for eculizumab, such as that submitted to the CAS Registry in 1999. In such a situation, it would have been the intention of the Applicant to disclose the sequence of eculizumab to the public in the patent application. However, the Applicant would not have been allowed to correct the sequence merely because it was his intention to make the correct sequence public and because the correct sequence was necessary to claim eculizumab and meet the sufficiency requirements of Article 83 EPC. To the contrary, the sequence in such a patent application could later be corrected only if it was immediately obvious that the sequence contained an error and also what the correct sequence should be (Rule 139 EPC). In other words, the Applicant's intent would not have been taken into consideration. We refer in this regard to Board of Appeal decision T1111/02 in which there were errors in a sequence provided in the patent application that led to a finding of insufficiency because the incorrect sequences could not be corrected under Rule 88 EPC (now Rule 139 EPC). Accordingly, it is the disclosure itself – rather than the intent of the Applicant – that is controlling. The same should apply in the present case (regardless of what the intent of the Applicant was in 1999).

The fact remains that the CAS database did not contain SEQ ID NOs: 2 and 4 of the present application at the filing date, and the incorrect sequences for eculizumab submitted to CAS in 1999 do not destroy the novelty of the present claims.

Eculizumab itself was not publicly available at the priority date

Eculizumab used in the clinical trials was not available to the public

The clinical trials described in the prior art did not make the antibody eculizumab available to the public. The position taken by the Examining Division in this regard is inconsistent with established jurisprudence of the Boards of Appeal.

We are filing herewith a new Declaration of Michael Bombara (D31) who managed many of the clinical trials that Alexion conducted in the late 1990s and early 2000s, and whose responsibilities included ensuring that all of the necessary contacts between Alexion and the principle investigators and/or their institutions or study sites were in place (see paragraph 2 of D31). Bombara confirms that no member of the public should have been able to obtain Alexion's confidential information and/or study drug material (eculizumab) in the course of any of the clinical studies (see paragraph 17 of D31).

The Bombara declaration summarises Alexion's clinical trial practice prior to the present filing date and explains the multiple layers of confidentiality that were built into each clinical study to ensure that the principal investigators and study

institutions or sites clearly understood their obligation to maintain Alexion's information, including the study drug, in strict confidence (see paragraph 5 of D31).

Bombara refers to one specific example of the agreements in place for the pilot PNH study of 11 PNH patients referenced in D1 to D5 (the "C02-001" study) and provides evidence of the agreements in place for this study. The declaration confirms that the authors on each of D1-D3 and D5 were affiliated with Alexion or one of the two institutions bound by these agreements (see paragraph 15 of D31). Bombara also states that similar documents would have been executed for all of the other eculizumab-related studies conducted by Alexion in the late 1990s and early 2000s (see paragraph 16 of D31).

The Bombara declaration additionally explains that the investigators and institutions agreed to adhere to strict requirements set forth in the clinical trial protocol regarding the tracking, logging, storage and disposal of study drug material, and provides an example of a drug disposal form (see paragraphs 12 and 13 of D31).

As an initial point, the Examining Division is incorrect that "*none of the documents cited in respect of the present application relate to clinical trials*". All of D1 to D5 relate to clinical trials carried out under the control of Alexion:

- D2 and D3 describe a pilot study that was conducted in 11 PNH patients for 12 weeks. This study is the "C02-001" study as identified in the table in the Boone declaration (D13) and Exhibit B of the Bombara declaration (D31).
- D5 describes an extension of the "C02-001" study during which the PNH patients were treated for 52 weeks. This extension study is the "E02-001" study identified in the table in the Boone declaration (D13) and Exhibit B of the Bombara declaration (D31).
- D1 describes a further extension of the "C02-001" study during which the PNH patients were treated for two years. This extension study is the "X03-001" study identified in the table in the Boone declaration (D13) and Exhibit B of the Bombara declaration (D31).
- D4, which discloses Alexion Pharmaceuticals, Inc. as its source (page 675, right column) is a review paper that describes a series of clinical trials that were conducted with eculizumab. All of these trials are studies identified in the table in the Boone declaration (D13) and Exhibit B of the Bombara declaration (D31):
    - The study in 117 patients with idiopathic membranous nephropathy is "C99-004".
    - The study in 40 rheumatoid arthritis patients is "C97-001".
    - The study in 209 rheumatoid arthritis patients is "C99-001".
    - The study in 368 rheumatoid arthritis patients is "C001-004".
    - The two month trial in 10 patients with dermatomyositis is "C99-006".
    - The phase II trial in membranous nephritis is "C99-004".
    - The trial in systemic lupus erythematosus is "C97-002".
    - The pilot and extension studies in 11 PNH patients are "C02-001" and "E02-001".

The Boone declaration (D13) explains that all of these trials were carried out under confidentiality agreements and that the eculizumab drug supplies used in the studies were tightly controlled. We have also provided direct evidence of the relevant

provisions in the "C02-002" and "C04-001" studies, by way of example. As is clear from paragraphs 7 to 9 of D13 and paragraphs 5 and 16 of D31, all of the studies listed in the table included substantively identical confidentiality provisions. It is also stated in paragraph 2 of the Bell declaration (D12) and paragraph 3 of the Bombara declaration (D31) that eculizumab was used in clinical trials before 15 March 2007 only under strict control and conditions of confidentiality.

The Examining Division is asking that we provide proof of a confidentiality agreement between Alexion and each participant/physician involved in the clinical studies. This level of proof is not, however necessary.

It is stated in Case Law of the Boards of Appeal III-G-5.1.1 that:

> "*In ex parte proceedings the applicant bears the burden of proof for the facts in his favour, e.g. the fact that a document cited by the examining division does not form part of the state of the art (**T 160/92**, OJ 1995, 35)*",

However, this statement does not require that the Applicant provide absolute proof. The Case Law of the Boards of Appeal III-G-5.1.1 goes on, in the following paragraph to say:

> "*As far as issues relating to patentability requirements are concerned, the burden of proof in **examination proceedings** cannot lie initially with the applicant. If an examining division raises an objection, it must be appropriately substantiated.* In **T 578/06** the *examining division had failed to provide such substantiation. Also, the passage in the "Case Law of the Boards of Appeal of the EPO" (see paragraph immediately above) could not support its view that in ex parte proceedings the applicant has the burden of proof for facts in his favour, as it appeared to have been taken out of its context. In this respect, the board clarified that all the procedural situations referred to in this passage were those where, in response to a substantiated objection from the examining division, the applicant was required to support its contention.*"

Further, the same section of the Case Law of the Boards of Appeal (III-G-5.1.1) goes on to explain that:

> "*Where a lack of novelty is alleged, the burden of proof invariably lies with the party claiming that the information in question was made available to the public before the relevant date (see, for example **T 193/84**, **T 73/86**, **T 162/87**, **T 293/87**, **T 381/87**, OJ 1990, 213; **T 245/88** and **T 82/90**). According to the boards' established case law, each of the parties to the proceedings bears the burden of proof for the facts it alleges. If a party, whose arguments rest on these alleged facts, does not discharge its burden of proof, this is to the detriment of that party, who may not shift the onus of proof onto the other party (see **T 270/90**, OJ 1993, 725; **T 355/97**; **T 836/02**; **T 176/04**; **T 175/09**; **T 443/09**).*"

We have provided direct evidence of confidentiality agreements in some of the studies using eculizumab (D22, D23 and D31), and declaratory evidence that confidentiality provisions applied to all of the studies using eculizumab (D12, D13 and

D31). The Examining Division, on the other hand, has not substantiated its arguments that eculizumab was made available to the public as a result of the clinical trials carried out before the filing date, nor has the Examining Division provided any contrary evidence to that we have submitted.

The evidence we have provided does not include any confidentiality agreements with the patients taking part in the clinical trials. However, the patients were never in a position to inspect or distribute eculizumab. Eculizumab is administered by intravenous infusion in the clinic (see Declaration of Leonard Bell (D12), paragraph 2 and Declaration of Michael Bombara (D31), paragraph 3). Therefore, the intravenous administration of eculizumab is akin to the implantation of a prototype device (T152/03 and T906/01) and the eculizumab clinical trials can be distinguished from the trials in T7/07 where a large number of patients were given tablets to take home with them for use over a longer period of time (see T7/07 Reason 3.3). The eculizumab used in the trials carried out prior to the filing date of the present application remained under the strict control of health care practitioners who were bound by strict confidentiality obligations and was not made available or accessible to the public.

Further, it is established in T152/03 and T906/01 that the development and test phases of implanted products were necessarily surrounded by secrecy as long as such products had not been commercialised, and that there was a prima facie assumption that any person involved in a medical tests of such products was obliged to maintain confidentiality and that any evidence proving the contrary was important. In the present case, eculizumab had not been commercialised at the filing date (see D13, paragraph 14, D15 and D16). There should therefore be an assumption that those involved with the trials were obliged to maintain confidentiality. Furthermore, we have provided evidence that the trials were carried out under confidentiality agreements with Alexion and that eculizumab used in the clinical trials remained under the strict control of Alexion (see D12, D13 and D31).

As stated in the Case Law of the Boards of Appeal, 8th edition, III-G-4.3.1:

> "*The EPO departments decide on the issues that arise before them on the basis of the evidence adduced by the parties. Their decisions need not, and in most cases cannot, be based on absolute conviction, but instead are to be arrived at on the basis of the overall balance of probabilities, in other words on the footing that one set of facts is more likely to be true than the other. If the result of the boards' evaluation of the evidence is such as to persuade them one way or another, then the* ***balance of probabilities*** *standard is met. This standard applies particularly in opposition appeal proceedings where the boards of appeal are called upon to reach a conclusion on the basis of the overall balance of probabilities, as distinct from "beyond all reasonable doubt" or "absolute conviction" (on the latter concept, refer to the more recent case* ***T 2451/13****). Each of the parties must therefore seek to prove facts alleged by it to that degree of proof (see e.g.* ***T 182/89****, OJ 1991, 391;* ***T 270/90****, OJ 1993, 725;* ***T 859/90****,* ***T 109/91****,* ***T 409/91****, OJ 1994, 653;* ***T 1054/92****,* ***T 296/93****, OJ 1995, 627;* ***T 326/93****,* ***T 343/95****,* ***T 363/96****). The standard of balance of probabilities applies also in decisions issued in ex parte proceedings (****T 381/87****, OJ 1990, 213;* ***T 69/86****,* ***T 128/87****, OJ 1989, 406;* ***T 939/92****, OJ 1996, 309).*

> *When an issue of fact is being examined and decided by the EPO on the balance of probabilities,* ***the more serious the issue*** *the more convincing must the evidence be to support it. If a decision on such an issue may result in refusal or revocation of a European patent, for example, in a case concerning alleged prior publication or prior use, the available evidence in relation to that issue must be very critically and strictly examined. A European patent should not be refused or revoked unless the grounds for refusal or revocation (that is, the legal and factual reasons) are fully and properly proved (****T 750/94****, OJ 1998, 32;* ***T 329/02****). See also Guidelines G-IV, 1 "State of the art – General remarks and definition" – November 2015 version): "If the applicant shows sound reasons for doubting whether the document forms part of the 'state of the art' in relation to his application and any further investigation does not produce evidence sufficient to remove that doubt, the examiner should not pursue the matter further."* [emphasis added]

The evidence we have submitted shows that the clinical trials using eculizumab were carried out under confidentiality agreements and that Alexion imposed strict controls on the eculizumab used in the trials. The eculizumab used in the trials was not available to the public. There was no public prior use of eculizumab, and the documents that describe the clinical trials that refer to eculizumab only by name (D1-D5, D10 and the patents and applications listed in D8 that have an earlier filing date that the present application) do not provide an enabling disclosure of eculizumab.

Use of the term "eculizumab" is not evidence that it was publicly available

The Examining Division is incorrect that the fact that many patent and non-patent documents in the prior art related to the use of "eculizumab" is evidence that the compound is well-known and available to the public.

It is established Board of Appeal case law that simply mentioning a biological material by name in a document does not make it publicly available. As stated by the Board in T576/91, if contractual obligations between parties resulted in access to biological material being deliberately restricted to a group of persons bound either by a research contract or a licence, it could not be concluded that this material had been made "available to the public" within the meaning of Article 54(2) EPC.

In this case, access to eculizumab was limited to the physicians involved in the trials who had signed confidentiality agreements with Alexion, agreed to store eculizumab in a secure, limited-access area and agreed to strict provisions regarding the tracking and disposal of any unused eculizumab. The mention of "eculizumab" by name in public reports of the trials and in patent applications is therefore not evidence that eculizumab was publicly available.

Thus, the absence of a statement in D1-D5 that eculizumab had not been "*available for public use or that the working group using it had been bound to a confidentiality agreement with Alexion Pharmaceuticals"* does not mean that the antibody was available to the public. The evidence shows that the working group using eculizumab was bound to a confidentiality agreement with Alexion Pharmaceuticals. Therefore,

in accordance with the case law (T576/91), it cannot be concluded that eculizumab had been made "available to the public" within the meaning of Article 54(2) EPC.

Articles 76(1) and 123(2) EPC

The amendment to refer to a light chain consisting of residues 23-214 of SEQ ID NO: 4, and to a light chain variable region consisting of residues 23-109 of SEQ ID NO: 3 is an obvious correction that meets with the requirements of Rule 139 EPC.

The Examining Division considers that:

> *"it cannot be excluded by the skilled reader that the SEQ ID NO: 3 and 4 as filed in the parent application and the application as originally filed were actually those for which protection was sought."*

The Examining Division notes that neither the original application nor the parent application contain a disclosure of the fact that SEQ ID NOs: 3 and 4 did not have to include the leader peptide. Such an indication is not, however, necessary because it was part of the skilled person's common general knowledge that antibodies do not include leader peptides. Evidence in support of the skilled person's common general knowledge has already been filed as D14, D19 and D20.

As established by the Enlarged Board of Appeal in G3/89 and G11/91:

> "T*he part of a European patent application or of a European patent relating to the disclosure (the description, claims and drawings) may be corrected under Rule 88, second sentence, EPC [now Rule 139, second sentence, EPC] only within the limit of what a skilled person would derive directly and unambiguously, using common general knowledge, and seen objectively and relative to the date of filing, from the whole of these documents as filed. Such a correction is of a strictly declaratory nature and thus does not infringe the prohibition of extension under Article 123(2) EPC."* (Head note 1; emphasis added)

The Examining Division talks in terms of protection being sought for SEQ ID NOs: 3 and 4. However, protection of these sequences is not what is sought. Rather, it is protection for an antibody defined in terms of its sequences that is sought by the Applicant. Therefore, it is not correction of SEQ ID NOs: 3 and 4 that is requested. Rather, correction is requested only of the definition of an antibody defined with reference to these sequences.

It would have been immediately apparent to the skilled person that the eculizumab light chain sequences shown in SEQ ID NOs: 3 and 4 of the original application and of the parent application include a 22 amino acid signal peptide at the N-terminus. The skilled person would know from his common general knowledge that the 22 amino acid signal peptide in SEQ ID NOs: 3 and 4 would be cleaved off during antibody production in the cell.

The skilled person would consequently immediately recognise that the recitation at page 5, lines 30-33 of the original application and of the parent application of an antibody that comprises a light chain that "*consists of SEQ ID NO: 4*" is an error, and

that the recitation at page 5, lines 25-28 of an antibody having a light chain variable region that "*consists of SEQ ID NO: 3*" is an error.

The skilled person, based on his common general knowledge, would also immediately know that it was the Applicant's intention to define the antibody as including only the mature light chain sequence, i.e. residues 23-214 of SEQ ID NO: 4 or residues 23-109 of SEQ ID NO: 3.

The correction to define the antibody only by reference to the mature light chain sequence is therefore an obvious correction in accordance with Rule 139 EPC, and the present application meets the requirements of Articles 76(1) and 123(2) EPC.

Sufficiency - Article 83 EPC

The insufficiency objection raised by the Examining Division arises because the Examining Division does not accept that the correction made to define the antibody in terms of the mature heavy and light chain sequences is an obvious correction of an obvious error. However, the Examining Division has misinterpreted the Applicant's arguments. The application does not include the wrong sequences. SEQ ID NOs: 3 and 4 are the correct light chain variable and full light chain sequences, respectively, but they contain the 22 amino acid signal peptide that is cleaved off during antibody production and so is not present in the mature antibody which comprises two heavy chains and two light chains. Accordingly, the sequences filed in the original application do identify the structure of eculizumab.

The question to ask when determining whether the disclosure of the application is sufficient is whether the skilled person could make the claimed antibody without undue burden based on the teaching in the patent.

Claim 1 defines the antibody by reference to its full mature heavy and light chain sequences. It is routine for the skilled person to make an antibody having known full mature heavy and light chain sequences. Therefore, there is no question that the skilled person would be able to make the antibody of claim 1 without undue burden.

Inventive Step - Article 56 EPC

The Examining Division has identified the difference between the "*available documents of the prior art describing eculizumab (D1-D5, D10 and D11)*" and the claimed antibody as being the sequence of the claimed compound. The Examining Division refers to the incorrect CAS database entry for eculizumab as was available at the filing date. It is argued that the contribution of the patent is correction of an error in a registered sequence listing, which is devoid of technical character. The Examining Division then asserts that the skilled person had the knowledge to "*carry out the sequence of eculizumab (see the teaching of D7)*". This is not the correct application of the problem and solution approach.

As discussed above, none of D1-D5, D7, D10 and D11 provide an enabling disclosure of "eculizumab". Therefore, the teachings of these documents in so far as they refer to "eculizumab" are not part of the state of the art under Article 54(2) EPC (T1437/05).

The present application is concerned with providing treatments for PNH. Therefore the closest prior art document is one that is also concerned with treating PNH.

D1 is concerned with treating PNH, and other haemolytic diseases, using inhibitors of complement components, such as complement-inhibiting antibodies. D1 describes a clinical trial using the specific anti-C5 antibody "eculizumab". D1 does not disclose the sequence of "eculizumab", but cites D7 and D11 as describing suitable anti-C5 antibodies including "eculizumab". As explained above, D11 does not disclose the full sequence of any antibody and the only full length antibody described in D7 is an IgG4 isotype antibody. Therefore, the teaching in D1 would have led the skilled person to an antibody having a naturally occurring IgG4 heavy chain constant region. Thus, whilst D1 does not provide an enabling disclosure of "eculizumab" for treating PNH, it does provide an enabling disclosure of the IgG4 antibody disclosed in D7 for treating PNH.

In contrast, the antibody of claim 1 has an engineered heavy chain constant region. The amino acid differences between the IgG4 heavy chain constant region and the engineered heavy chain constant region of the antibody of claim 1 are shown in the Bell declaration (D12) in paragraph 5. The technical effects of these differences are also set out in D12. Paragraph 6 of D12 explains that differences in the heavy chain constant region result in the antibody having reduced binding to Fcγ receptors such that immune responses that are undesirable for an antibody intended to shut down the terminal complement pathway are minimised (see also the experimental report, D18). Paragraph 7 of D12 explains that differences in the heavy chain constant region result in the antibody having an increased plasma half-life (see also the experimental report attached as D29).

The objective technical problem in view of D1 may therefore be defined as the provision of an antibody having improved properties compared to the IgG4 antibody of D7 for therapeutic use in treating PNH. The solution is solved by the presently claimed antibody. The solution provided would not have been obvious to the skilled person at the filing date.

D7 explains why the IgG4 isotype was chosen for the full length antibody, reporting that:

> "*The human IgG4 isotype was chosen, as this isotype does not activate human complement (Tao et al., 1993) and there is only one known allotype of IgG4 (Ghanem et al., 1988), precluding the potential development of allo-antibodies in patients. The humanized h5G1.1 (CDR) HuG4 antibody bound to human C5 with a similar avidity as the murine antibody when assayed by ELISA (Fig. 9) and inhibited lysis of porcine aortic endothelial cells as effectively as the murine antibody, with a 1:1 molar ratio of antibody binding sites to human C5 being sufficient for inhibition (Fig. 10)*" (D7, page 1399, right column, second paragraph, lines 15-25)."

The skilled person would have had no motivation to use an antibody different than the IgG4 antibody described in D1/D7 in view of the report in D1 of the successful treatment of PNH, and the teaching in D7 discussed above that the human IgG4 isotype has the advantages that it does not activate human complement and has only

one known allotype, precluding an immune response against the constant region of the antibody.

In particular, the skilled person would not consider using a different, engineered constant region sequence in place of the IgG4 isotype antibody disclosed in D7 because he would be concerned about the immunogenicity of the therapeutic antibody, and D7 teaches that the IgG4 isotype is ideal in this regard. The safety of the IgG4 antibody of D7 would have been confirmed to a skilled person by the clinical studies reporting that "eculizumab" (identified as the antibody of D7) was safe and effective when administered to PNH patients (e.g. D1, D3 and D4). Also, at the filing date of the application, other IgG4 isotype antibodies had been commercialised as therapeutic antibody products (see D30, table 1). Significantly, at the filing date of the application, no antibody having the uniquely-engineered heavy chain constant region of SEQ ID NO: 2 was on the market or publicly known to be studied in human clinical trials.

Furthermore, the advantageous properties of reduced immunogenicity and increased half-life exhibited by the claimed antibody are not taught or suggested in any of the cited documents. Indeed, in paragraph 8 of D12, it is explained that the advantageous properties of the claimed antibody over the antibody of D7 (which is the antibody to which D1 relates) were surprising and unpredictable.

Accordingly, the solution to the problem of providing an antibody having improved properties compared to the IgG4 antibody of D7 for therapeutic use in treating PNH provided by the present claims would not have been obvious to the skilled person at the filing date, and the claims involve an inventive step.

First Auxiliary Request

In the first Auxiliary Request, the antibody is defined as "*comprising the heavy chain amino acid shown in SEQ ID NO: 2 and the light chain amino acid sequence shown in SEQ ID NO: 4*". Basis for this amendment may be found in the originally filed application at page 4, line 32 and page 44.

Second Auxiliary Request

The second Auxiliary Request is the same as the Main Request, except that claim 1 has been rewritten as a purpose-limited product claim reciting the treatment of PNH.

Third Auxiliary Request

The third Auxiliary Request contains the amendments made to the first and second Auxiliary Requests.

Conclusion

It is believed that this application is in allowable form and the Examining Division is requested to reconsider it favourably. The Applicant has addressed each and every issue identified by the Examining Division in the Summons to Oral Proceedings. The Applicant would appreciate it if the Examining Division would consider the response as soon as possible and provide feedback. It is hoped that the application can be

allowed without the need for Oral Proceedings on 5 September 2019, on the basis of the Main Request or, failing that, on one of the Auxiliary Requests. Accordingly, the Examiner is requested to telephone the undersigned once the Examining Division has considered our submissions.

I look forward to hearing from the Examiner.

Yours faithfully

Electronically Signed
DR PAMELA M TUXWORTH
ASSOCIATION NO. 811

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URGENT! PLEASE FORWARD IMMEDIATELY TO THE EXAMINING DIVISION!
ORAL PROCEEDINGS SCHEDULED FOR SEPTEMBER 5, 2019

September 2, 2019

Third Party Observations

Application Number	16 195 355.9 - 1466
Publication Number	EP 3 167 888
Title	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT
Applicant	Alexion Pharmaceuticals, Inc.
Our file reference	AMG17203ADV-EP888

Third Party Observations

We would like to respectfully bring the following Third Party Observations to the Examining Division's attention:

The Applicant acted against the well-established principle of *nemo potest venire contra factum proprium*:

(1) In their submission dated August 15, 2018, Alexion attempt to defend their contradictive behavior in the proceedings regarding EP 1 720 571 and the current application. The Applicant's representative asserts that the principle of *nemo potest venire contra factum proprium* would not be applicable in this case (cf. p. 6, ultimate par. of submission dated August 15, 2018). This is incorrect.

(2) The principle of the protection of legitimate expectations is a general principle well established in European Community law and generally recognized in the EPC contracting states and Board of Appeal case law (**G 2/97**, OJ 1999, 123; see also **R 4/09**). The term "good faith" is also used to describe this concept (**J 10/84**, OJ 1985, 71; **J 38/97**). Hence, it is established case law that users of the European patent system, who are

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8 Rechtsanwalt

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parties in proceedings before the EPO, must act in good faith (**G 2/97**, **R 4/09**).

(3) According to the "Saxe Declaration" submitted by the Applicant on January 16, 2018, Stephen Saxe submitted a letter to CAS dated April 28, 2009 explaining:

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

(4) Thus, Alexion were aware that they provided incorrect information regarding eculizumab to CAS at least as of April 28, 2009. Contrary to the Applicant's assertion, when filing the response of December 30, 2013 in the EP'571 case, Alexion thus did not make a *"mere assertion that turned out to be erroneous"* (cf. p. 6, 4th par. of submission dated August 15, 2018). Rather, they made an assertion and at the same time apparently assumed that the contrary was the case.

(5) In their submission dated July 5, 2019, Alexion argue that the copy of the CAS entry, provided with their letter in the opposition proceedings regarding EP 1 720 571 did not contain any sequences, that the identity of the sequences was not investigated, and that the argument was not challenged at that time (cf. p. 6, 6th to 9th par. of letter dated July 5, 2019). We note that none of these allegations take away from the fact that Alexion have asserted before the OD in the EP'571 case that the sequence of eculizumab had been submitted to CAS knowing that this was incorrect.

(6) Alexion further try to defend themselves by arguing that they did not gain any advantage from their knowingly false statement (cf. p. 7, 1st par. of letter dated July 5, 2019). Of course, the obligation to act in good faith does not depend on the question whether a party gains an advantage or not.

(7) On the other hand, Alexion did not provide any evidence that they indeed filed the wrong structural information to CAS, since the Saxe Declaration - contrary to the assertion of Alexion's representative - does not include said "wrong" structure or any excerpt of the CAS registry showing that a "wrong" structure was submitted there. With the letter dated July 5, 2019, Alexion filed an excerpt from a CAS online resource allegedly showing the sequences originally submitted to CAS (**D28**). We note that the document provided by the Applicant does not include any information about the date on which the sequence shown therein has been submitted to CAS and/or been amended. This document, apparently retrieved on June 11, 2019, is therefore

unable to prove that the incorrect sequence has been submitted to CAS in 1999, i.e., twenty years ago.

(8) In this regard we would like to point out that the Applicant's allegation – that the patents and applications cited in **D8** would refer to anti-C5 antibodies more generally and that it would, therefore, be of no importance whether or not the correct sequence of eculizumab had been disclosed (p. 8, 1st to 3rd full paragraph of Applicants submission of August 15, 2018) - is simply not tenable. Rather, the question whether or not the correct sequence of the antibody designated via the INN eculizumab was available at CAS – published and provided with a CAS registry number - was of utmost importance.

(9) An INN is an **official** generic and non-proprietary **name** given to an active ingredient. The INN "eculizumab" is the **official** generic **name** given to the substance having the CAS-Number 219685-50-4. In conclusion, the above presented facts inevitably imply that the public – in particular authors of scientific literature but also the EPO - could rely upon the fact that a correct description of eculizumab was publicly available at the priority date.

(10) As will be explained below, during prosecution of EP 1 720 571 B1 and EP 2 359 834 B1, the Examining Division considered the term "eculizumab" at all times to disclose the specific antibody related to said INN. Alexion's representatives took the same position, even though Alexion apparently assumed at least as of April 2009 that the INN eculizumab related to a false structure (as they allege now).

(11) Claim 1 of EP 1 720 571 B1 as granted directly relates to eculizumab. It is readily derivable from the prosecution history (see first office action dated September 2, 2009 (enclosed as **Annex I**), Items 4.1 to 4.3 of the Communication; Summons to Oral Proceedings dated March 4, 2011 (enclosed as **Annex II**), Items 2.1, 5.1, 6.1) that the Examining Division heavily relied upon the fact that eculizumab was known on the priority date. In the Summons to Oral Proceedings the Examining Division further stated under Item 3.2 that:

> *"The application provides experimental details of dosage optimization of an anti-C5 antibody, 5G1.1* ***eculizumab****, for the treatment of PNH in a group of 11 patients.* ***Given the different affinities, functions and pharmacokinetics of individual, specific antibodies, the establishment of an optimal dosage schedule will be dependent on the antibody used****. Since independent claims 1-3 and 8-10 do not contain this feature it does not meet the requirement following from Article 84 EPC, taken in combination with Rule 43(1) and (3) EPC, that any independent claim must contain all the technical features essential to the definition of the invention."*

(12) Although Alexion assumed that eculizumab was not disclosed at the priority date, as they now allege, the disclosure of eculizumab was confirmed by them vis-à-vis the Examining Division (see reply to Summons to Oral Proceedings dated September 23, 2011 (enclosed as **Annex III**), page 9, second paragraph). Because the application as filed did not disclose the actual structure of eculizumab this was the only way to overcome the cited objection.

(13) The sole claim of EP 2 359 834 B1 as granted directly relates to eculizumab. Again, the Examining Division heavily relied upon the notion that eculizumab had been disclosed before the priority date (see e.g. European Search Opinion dated July 21, 2011 (enclosed as **Annex IV**), objection under Item 2, upheld by the Examining Division in further prosecution). The prior disclosure of eculizumab was also confirmed by Alexion *vis-à-vis* the Examining Division (see reply to Office Action dated November 15, 2012 (enclosed as **Annex V**), Item 2.2.2):

> *"This understanding is further underlined by the general knowledge in the art of antibody engineering and the skilled person's understanding of the structural features of eculizumab, for example, which was known to comprise "a G2/G4 [constant region] in place of the G1 domain" (see specification at page 5, lines 16 to 18; see also the package insert for Soliris®, the trade name under which eculizumab is sold, at paragraph 11 (a copy of the package insert is submitted herewith as Exhibit A)). Applicant also provides Mueller et al. (1997) Mol Immunol 34(6):441-452 as Exhibit B, which describes a G2/G4 Fc portion of an antibody as lacking complement activating activity (see, e.g., page 446, column 2 and Fig. 9)."* (Emphasis added.)

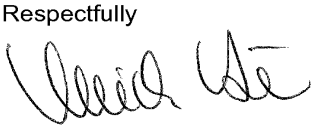
(14) Again, Alexion relied upon the public's knowledge of the structure of eculizumab before the Examining Division to support the patentability of claims to "eculizumab" but now assert that one of skill in the art lacked such knowledge.

(15) In summary, the WHO named eculizumab to be the official generic name given to the substance having the CAS-Number 219685-50-4. According to their own statements, Alexion were aware of having provided incorrect information regarding eculizumab to CAS at least as of April 28, 2009. Therefore, the concealment of this information cannot be considered to merely have been erroneous. By concealing this information Alexion cannot be said to have acted in good faith when attempting to overcome the various objections from the Examining Division, which objections could not have been overcome had the EPO been informed that the INN eculizumab was in fact assigned to a false structure. On the other hand, there is no evidence provided that Alexion have indeed filed "wrong" structural information to CAS. At the moment, this appears to be a mere assertion.

(16) In conclusion, Alexion's conduct vis-à-vis the EPO is contrary to the principle of *nemo potest venire contra factum proprium*, and their attempt to justify their concealment of alleged facts, which, if true would have been critical for validity in a number of proceedings, and which are

nonetheless now supposed to serve as basis to restore novelty over prior art documents, must be refused.

Respectfully



Dr. H. Ulrich Dörries

Enclosures
Annexes I-V

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Our ref: SK/6930 Your ref: **EP 3 167 888** Wroclaw, September 4th, 2019

Re: **Third Party Observations in respect of EP 3 167 888**

THIRD PARTY OBSERVATIONS

European Patent:	Application Number: EP 16195355.9
	Publication Number: EP 3 167 888
Applicant:	Alexion Pharmaceuticals, Inc.
Title:	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

Dear Sirs,

We enclose the Third Party Observations in respect of EP 3 167 888 for attention of Examining Division.

Your faithfully

Rafał Witek
Patent Attorney

Enclosures:

1. Third Party Observations in respect of EP 3 167 888
2. EP1558648_Request for correction
3. EP1558648_Original filing (WO 2004/035607 A2)
4. EP1558648_Vink declaration
5. EP2581113_Request for correction
6. EP2581113_Application as filed

4th September, 2019

EP Appl. No. 16 195 355.9 – 1466 (EP 3 167 888)
In the name of Alexion Pharmaceuticals, Inc.

Third Party Observations

We would like to respectfully bring the following Third Party Observations to the Examining Division's attention:

I. The amended claims submitted by the Applicant as Main Request contravene Articles 123(2) and 76(1) EPC

(1) In response to the Examining Division's Communication in preparation of oral proceedings, dated May 7, 2018, the Applicant argued in submissions dated August 15, 2018 and July 5, 2019, that the skilled person would have immediately recognized that the Applicant could not have intended to seek protection for an antibody comprising all of SEQ ID NO: 3 or all of SEQ ID NO: 4 because he/she would immediately have seen that these sequences contain a signal peptide (leader sequence) and would know that it is not possible to make a mature antibody that contains a signal peptide.

(2) The Applicant pointed to Enlarged Board of Appeal decisions **G 3/89** and **G 11/91** to support his allegations. We note that the Applicant's arguments are based on a flawed interpretation of the cited case law.

(3) A correction under Rule 139 EPC is of a strictly declaratory nature. Two requirements arise for a correction under Rule 139 to be allowable, i.e., not to contravene Article 123(2) EPC and/or Article 76(1) EPC. First, the presence of an error must be obvious. Second, it has to be *"immediately evident [for a skilled person] that nothing else would have been intended than what is offered as the correction"* (Rule 139 EPC, second sentence). If there is any doubt, including even slight ambivalence, that nothing else would have been intended than what is offered as the correction (i.e., the correct sequence of eculizumab), a correction cannot be allowed (see also **J 16/13**, points 8 to 10 of the reasons).

(4) The Proprietor has not met these requirements as they have not shown that it would be immediately evident that the leader sequence of SEQ ID NOs: 3 and 4 would precisely consist of residues 1-22 of SEQ ID NOs: 3 and 4 and nothing else.

(5) The Proprietor alleges in this regard (par. bridging p. 2/3 of the submission dated August 15, 2018) that the skilled person would know from his common general knowledge that the *"framework of human kappa light chains commonly starts with the residues Asp Ile Gln Met as shown in the Table at pages 103 to 108 of Kabat et al."* (Exhibit E filed with the Andrien Declaration on July 10, 2017). Notably, the table

of contents (Exhibit C filed with the Andrien Declaration on July 10, 2017) makes clear that the Applicant chose to submit only a fraction of the human kappa light chain sequences disclosed in Kabat *et al.* (according to the table of contents, human kappa light chain sequences are disclosed on pages 103 to 130 of Kabat *et al.* out of which only pages 103 to 108 have been submitted). The sequence variability among human kappa light chains must therefore be assumed to be considerably greater than what is derivable from the documents submitted by the Applicant.

(6) However, even within the limited selection presented by the Applicant, Asp Ile Gln Met are at best commonly the first residues. When assessing the allowability of an amendment under Rule 139 EPC, the question is not whether human kappa light chains commonly start with any particular sequence, but whether they must start with a certain sequence and whether this would have been obvious to the skilled person. Otherwise, the skilled person would be left with uncertainty, at which specific residue of SEQ IDs NO: 3 and 4 the correct mature sequence (excluding the leader peptide) would begin. This is clearly the case here, which will we explained in more detail below.

(7) Specifically, one question is how many residues the skilled person would have subtracted from the sequence disclosed in the patent, if recognizing that said sequences comprise a leader peptide? Further, how would the skilled person arrive at one and only one possible sequence to deduct from SEQ ID NOs: 3 and 4? The Proprietor states that *"the last two residues [of the leader peptides] are predominantly Arg Cys"* (par. bridging p. 2/3 of the submission). Again, as the Examining Division will be aware, *"predominantly"* does not meet the standard required for a correction under Rule 139 EPC. Quite to the contrary, the fact that the Applicant uses the term "predominantly" already shows that there are alternatives to the correction that would be apparent to the skilled reader of the application. However, as noted above, there must not be any ambiguity for a correction to be allowable. Otherwise, the skilled person would be confronted by the correction with subject matter that is not originally disclosed.

(8) The Applicant thus failed to show that a skilled artisan would immediately recognize how to replace the incorrect sequences (i.e., what the specific sequence of the leader peptide would be), with certainty.

(9) The Applicant's representative further asserted that similar requests for corrections would have been accepted by the EPO, pointing to EP 1 558 648 and EP 2 581 113. Regarding EP 1 558 648, the relevant protein sequences as well as the CDR sequences were derived by standard sequencing procedures (Example 2, WO 2004/035607 A2, submitted herewith). The antibodies were then claimed by reference to V_L and V_H sequences or to CDR sequences which is also standard in the art. Moreover, the original application disclosed at p. 29, I. 35-36 that *"heavy and light chain leader sequences are cleaved during protein maturation and do not contribute to the properties of the final antibody"*.

(10) The Applicant of EP 2 581 113, had pointed to page 67, paragraphs [0222] and [0223] of the application as filed (submitted herewith), disclosing that the presence of a signal sequence in a VL or VH amino acid

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sequence can be determined by comparing the length of the VL or VH amino acid sequence with those of known antibodies.

(11) Both applications referred to by the Applicant therefore discuss the occurrence of leader sequences or a clear identification of the mature antibody by comparison to known peptides. As pointed out above, the current application as filed is completely silent about leader sequences and does not provide any guidance for the skilled person how to clearly identify the claimed antibody. Therefore, these cases cannot be compared to the present one.

(12) Notably, with the request for correction of an obvious error, the Applicant in the EP 1 558 648 case submitted an expert declaration of Tom Vink (attached hereto). In the declaration, the expert explains the following (section 10 of the Vink declaration):

> 10. The leader sequence for a V_H sequence is typically 19-21 amino acids long, and the leader sequence for a V_L kappa sequence is typically 19-23 amino acids long. A protein always starts with a Met residue, so the leader sequences always start with a Met residue. A V_H sequence may start with a Glu or Gln residue, a V_L kappa sequence may start with Ala, Asp, Val, Asn or Glu. V_H signal peptides can end with Cys, Ala, Ser or Pro, and V_L kappa signal peptides can end with Cys, Ala, Gly or Glu. Accordingly, a person skilled in the art would know from studying the nucleotide and amino acid sequences (SEQ ID Nos: 1-12) that the leader sequences are indeed included. Also a person skilled in the art would note that the amino acid sequences are longer than usual for the mature amino acid sequences. This information was known to a person skilled in the art at the earliest priority date of the application and further supports that the leader sequences are indeed included in the variable heavy chain and light chain amino acid sequences as identified by SEQ ID Nos: 2, 6, 10, 4, 8 and 12, respectively.

(13) According to the statement of the expert, V_L kappa signal peptides typically have a length of 19 to 23 AAs and end with C, A, G or E, while human V_L kappa chains begin with A, D, V, N or E. We note that the number of possible combinations appears to be even larger, when taking Kabat *et al.* into regard (see above). However, if this reasoning is applied to the sequences disclosed in EP 3 167 888 as filed originally (see below) at least one alternative cleavage site to the one suggested by Alexion would be apparent to the skilled person, namely between amino acids 19 and 20 (WLR**GA**RC). The resulting leader peptide would be 19 amino acids in length, end with a G residue and the resulting mature V_L chain would begin with an A residue. Thus, all requirements that the skilled person would allegedly be aware of (according to the Vink declaration) would be fulfilled.

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SEQ ID NO: 3 – Eculizumab V_L

MDMRVPAQLLGLLLLWLRGARCDIQMTQSPSSLSASVGDRVTITCGASENIYGAL
NWYQQKPGKAPKLLIYGATNLADGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQ
NVLNTPLTFGQGTKVEIKRT

SEQ ID NO: 4 – Eculizumab Light chain

MDMRVPAQLLGLLLLWLRGARCDIQMTQSPSSLSASVGDRVTITCGASENIYGAL
NWYQQKPGKAPKLLIYGATNLADGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQ
NVLNTPLTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKV
QWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG
LSSPVTKSFNRGEC

(14) This is clear evidence that the skilled artisan would in the present case at the very least be confronted with two alternatives, when recognizing that SEQ IDs NO: 3 and 4 of the current application as filed erroneously comprise a leader peptide. Under such circumstances, the correction under Rule 139 EPC suggested by the Applicant would inevitably add information that is not originally disclosed.

(15) We lastly note that the *Kabat et al.* reference, as submitted by the Applicant, represents only a small (and potentially purposeful) selection from an amino acid sequence compendium that itself represents only a selection from the vast space of protein, or antibody sequences. It cannot provide evidence that nothing else than the sequences disclosed therein would have been thinkable sequences of signal peptides, or mature human kappa light chains for the skilled person.

(16) This is aggravated by the fact that the 5th edition of Kabat *et al.* was published in 1991, i.e., 15 years prior to the priority date of the application. In the quickly moving field of pharmacologically active antibodies, the space of known antibody sequences has significantly expanded within this period and thus, the sequence compendium referred to by the Applicant was long outdated at the priority date. The number of mature light chain sequences known in the art was certainly much larger in 2006, compared to 1991, accordingly, the number of possible options for the length and/or sequences of signal peptides was likely much larger in 2006, compared to 1991. Thus, the skilled person would have been confronted with even more uncertainty regarding the sequence and length of the signal peptide comprised in SEQ ID NOs: 3 and 4, even if recognizing that the sequences disclosed therein are immature.

(17) Thus, Kabat *et al.* is anyway not representative of the entire space of possible sequences the skilled person would have had to consider at the relevant date.

(18) In summary, the application provides no suggestion that specifically residues 1-22 of SEQ IDs NO: 3 and 4 are necessarily leader sequences, let alone that nothing else would have been intended than what the Applicant offered as correction. It can by no means be assumed that it would have been apparent to the skilled person that the sequence submitted by the Applicant is the only conceivable replacement for the sequences originally disclosed.

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(19) Therefore, the Applicant's request for correction of the description and the reference to residues 23 to 214 of SEQ ID NO:4, introduced into claim 1 of the MR contravene Articles 123(2) and 76(1) EPC.

II. The Auxiliary Requests filed on July 5, 2019

(20) With the submission dated July 5, 2019, the Applicant has submitted three Auxiliary Requests, AR1 to AR3. The Auxiliary Requests not only fail to overcome the objections raised by the Examining Division, they also introduce further violations of the EPC. This is explained in further detail below.

II.1 Auxiliary Request 1

(21) AR1 defines the claimed antibody by reference to SEQ ID NOs 2 and 4, however, the claim language has been amended such that claim 1 relates to an *"antibody that binds C5 comprising the heavy chain amino acid sequence shown in SEQ ID NO: 2 and the light chain amino acid sequence shown in SEQ ID NO: 4"* instead of *"comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4"*.

(22) This alternative claim language is a clear violation of Articles 76(1) and 123(2) EPC. The application as filed refers to an antibody comprising a heavy chain and a light chain consisting of SEQ ID NOs: 2 and 4, respectively (cf. application as filed at p. 5, li. 30 to 33). In contrast, claim 1 of AR1 refers to an antibody which comprises the heavy and light chain sequences "shown in" SEQ ID NOs 2 and 4. The Applicant thus effectively replaced "consisting of" by "comprising", which in itself represents a violation of Articles 76(1) and 123(2) EPC, as the terms are not freely interchangeable (**T 759/10**).

(23) Thus, while the application as filed (and previous claim 1) defines the antibody in a closed manner (heavy and light chains consisting of SEQ ID NOs 2 and 4), claim 1 of AR1 encompasses antibodies that comprise the sequences "shown in" SEQ ID NOs 2 and 4, but may, due to the open claim language, also comprise further sequence elements. For example, an antibody comprising a heavy and a light chain, wherein the heavy chain comprises the amino acid sequence of SEQ ID NO: 2 elongated by a few further amino acids at the C-terminus would fall under claim 1 of AR1. The original disclosure does not teach such modified antibodies.

(24) The alleged basis provided by the Applicant in the letter dated July 5, 2019 (original application, p. 4, li. 32 and p. 44) fails to disclose the subject matter of claim 1 of AR1. The description text of the parent application as filed and of the divisional application as filed are identically worded. Page 4, line 32 of the original application teaches that *"[i]n certain embodiments, said antibody is eculizumab"* (emphasis added). This passage neither provides basis for the "comprising" language, nor for the vague "shown in" phrase of claim 1 of AR1. Page 44 of the application discloses the (partially incorrect) SEQ IDs. Taken together, these two passages indicate to the skilled person that the antibody disclosed in the application may be an antibody named "eculizumab", which consists of heavy chains and light chains, wherein the

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heavy and light chains consist of the sequences disclosed as SEQ ID NOs 2 and 4. A widening of this concept through the use of "comprising" language cannot be based on this disclosure.

(25) In addition, the introduction of the term "shown in" leads to a further violation of Articles 76(1) and 123(2) EPC. This is because different interpretations of this phrase are possible, at least some of which would introduce subject matter that lacks original disclosure. If *"the light chain amino acid sequence shown in SEQ ID NO: 4"* means "the light chain amino acid sequence of SEQ ID NO: 4", claim 1 of AR1 would refer to an antibody that is not eculizumab, and thus, an antibody for which protection is not actually sought, as explicitly stated by the Applicant (e.g., letter of July 5, 2019, p. 15, 1st full par.). The amendment would furthermore not solve the objections of the ED with regard to insufficiency of disclosure.

(26) Alternatively, the term "shown in" could be interpreted as meaning any light chain sequence(s) comprised in SEQ ID NO: 4. It may be assumed that this is the interpretation desired by the Applicant, because this would enable him to *inter alia* cover the (correct) light chain sequence of eculizumab via reference to the (incorrect) sequence disclosed in the application text (SEQ ID NO: 4). However, in this case, too, the amendment would not solve the objections of the ED with regard to insufficiency of disclosure, since a light chain amino acid sequence of SEQ ID NO: 4 would still be encompassed. Further, since SEQ ID NO: 4 encompasses at the very least two potential light chain sequences when interpreted in light of common general knowledge (cf. above), this claim interpretation would inevitably lead to the finding that claim 1 adds matter, since it now encompasses several (potential) light chain sequences, instead of one clearly defined light chain sequence.

(27) The introduction of the vague term "shown in" furthermore renders claim 1 wholly unclear. As explained above, it is not clear to the skilled person which exact sequence(s) are encompassed by claim 1 of AR1. The heavy and light chain sequences of SEQ ID NOs 2 and 4, or any heavy and light chain sequence(s) that are part of SEQ ID NOs 2 and 4, i.e., any sequence fragment that can be extracted from SEQ ID NOs 2 and 4 and that may serve as an antibody heavy, or light chain, respectively - together with further derivatives thereof comprising additional amino acids?

(28) In summary, AR1 violates at least Articles 84, 76(1) and 123(2) EPC.

II.2 Auxiliary Request 2

(29) Claim 1 of AR2 defines the claimed antibody by reference to *inter alia "residues 23 to 214 of SEQ ID NO: 4"*. As explained above, this reference to only part of SEQ ID NO: 4 is not derivable from the application as filed. Claim 1 of AR2 therefore suffers from the same deficiencies under Article 76(1) and 123(2) EPC as claim 1 of the Main Request.

II.3 Auxiliary Request 3

(30) AR3 is based on AR1, with claims 1 and 2 being reformulated to medical use claims by addition of the phrase *"for use in treating paroxysmal nocturnal hemoglobinuria"* to claims 1 and 2. Claim 2 is further

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amended by introduction of the definition of the antibody recited in claim 1. AR3 inherits the deficiencies under Article 76(1), 123(2) EPC and 84 EPC put forward above with regard to AR1.

Encl.
EP1558648_Request for correction
EP1558648_Original filing (WO 2004/035607 A2)
EP1558648_Vink declaration
EP2581113_Request for correction
EP2581113_Application as filed

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Therapeutics and Clinical Risk Management

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REVIEW

Eculizumab in the management of paroxysmal nocturnal hemoglobinuria: patient selection and special considerations

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Abstract: Paroxysmal nocturnal hemoglobinuria (PNH) is a nonmalignant clonal disorder resulting from somatic mutation in the *PIG-A* gene leading to a deficiency of the membrane-anchoring molecule glycosylphosphatidylinositol. The lack of expression of two glycosylphosphatidylinositol-anchored proteins involved in the regulation of the complement system renders PNH erythrocytes susceptible to complement-mediated lysis. Clinical manifestations include thromboembolic disease, chronic kidney injury, pulmonary hypertension, smooth muscle dysfunction, and chronic hemolysis. Until recently, treatment was mainly supportive with most patients suffering from significant morbidity and shortened survival compared to age-matched controls. The development of eculizumab, a humanized monoclonal antibody directed against the terminal complement protein C5, has resulted in dramatic improvements of survival and reduction in complications. In this paper, we review some special considerations pertaining to the use of eculizumab for PNH.

Keywords: somatic mutation, GPI, survival, hemolysis, MDS, anemia

Overview of paroxysmal nocturnal hemoglobinuria

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hemolytic anemia resulting from a somatic mutation in the *PIG-A* gene in the hematopoietic stem cells.[1] This mutation leads to a deficiency of glycosylphosphatidylinositol (GPI) membrane-anchoring proteins on the surface of PNH cells. Two of these anchoring proteins, CD55 and CD59, are complement inhibitors, and the deficiency of such proteins will render PNH erythrocytes susceptible to complement-mediated lysis.[2] Recently, new evidence on PNH pathogenesis supports the notion that the expansion of the PNH clone results from escape from T-cell-mediated autoimmune damage to hematopoietic stem cells, with the GPI molecule as a target. This might further consolidate the observed association between PNH and aplastic anemia (AA).[3] PNH is a very rare disease with a prevalence of ~1–6 per million people,[4] and it can present at any age but mainly occurs in young adults, with the usual age at diagnosis being early 30s.[4–6]

Despite being rare, PNH is "the most vicious acquired thrombophilic state known", yet the mechanism of this virulent thrombotic behavior is still elusive.[3,7] Thromboembolic (TE) events occur in ~50% of PNH patients, and it is the main cause of death in approximately one-third of patients with PNH.[8] The intravascular hemolysis in PNH is a critical factor in triggering thrombosis. The release of hemoglobin (Hb) into the blood stream results in massive nitric oxide scavenging leading to vasospasm and to increase in platelets' reactivity and aggregation.[9,10] Therefore, the process of nitric

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oxide depletion by the free Hb will not only predispose patients to developing thromboembolism but also lead to vascular constriction (manifested as pulmonary hypertension, renal dysfunction, and erectile dysfunction) and smooth muscle dystonia of the gastrointestinal tract (manifested as abdominal pain, dysphagia, and esophageal spasm).[2,9,11] Additionally, renal dysfunction is also caused by chronic hemolysis-induced hemosiderin deposition in the proximal renal tubules, in addition to alterations in renal blood flow caused by nitric oxide depletion.[9,10]

Until a decade ago, the treatment options for PNH were mainly supportive including blood transfusion, erythropoiesis-stimulating agents, corticosteroids, anabolic steroids, iron therapy, thrombosis prophylaxis, and sometimes thrombolytic therapy or allogenic bone marrow transplantation.[2,3] Eculizumab was first introduced in 2004 in a preliminary pilot study of eleven participants.[12] It was found to dramatically reduce intravascular hemolysis and blood transfusion requirements. Despite the open-label design, the potential risk of bias was minimal as all the reported outcomes were objectively measurable. However, other issues including the small sample size, short-term follow-up, and the potential risk of selection bias (only transfusion-dependent patients were included) should be considered when interpreting the results of this study. Subsequently, two Phase III trials – the TRIUMPH and SHEPHERD studies – proved that eculizumab therapy for PNH is effective, safe, and well tolerated in a standardized setting with predefined outcome criteria.[11,13] A total of 87 and 97 patients were enrolled in these studies, respectively, overcoming the small sample size issue of the pilot study. However, issues such as selection of only transfusion-dependent patients and failure to report on TE rates as most patients were receiving concomitant anticoagulants remained. Eculizumab was finally approved for the treatment of patients with PNH in 2007 by the US Food and Drug Administration and by the European Medicines Agency.[2,8]

In this review, we focus on the up-to-date established positive impacts of eculizumab on PNH and its potential adverse events, highlighting certain issues that need to be addressed with eculizumab use.

Eculizumab

Eculizumab is a humanized monoclonal antibody directed against the terminal complement protein C5, which blocks the formation of the membrane attack complex (C5–9), thus protecting PNH red blood cells from complement-mediated intravascular hemolysis.[3,14] Its effectiveness has been evidenced by reduction or elimination of the need for blood transfusion, decrease in the incidence of thrombosis, improvement in anemia and quality of life (QoL), and prolongation of overall survival (OS).[1,8,11] Furthermore, due to its use, stem cell transplantation is only considered for cases of severe marrow failure.[3,15]

Pharmacodynamics and pharmacokinetics

An inhibition of C5 sufficient to prevent hemolysis occurs at eculizumab plasma concentrations in excess of 35 g/mL.[2] At therapeutic doses, maximum plasma concentrations of eculizumab are achieved within 1 hour of infusion.[2] Eculizumab reduces hemolysis in ~1 week, and its half-life elimination is ~11 days (range: 8–15 days). The clearance for a typical PNH patient weighing 70 kg is ~22 mL/h, and the volume of distribution is ~7.7 L.[16]

Dose and schedule

For PNH, eculizumab is administered as a weekly dose of 600 mg by intravenous infusion for 35 minutes from week 1 to week 4, followed by infusion of 900 mg at week 5, and then 900 mg every 14 days for an indefinite period.[1,2]

Eculizumab and PNH – clinical efficacy

The effect of eculizumab on PNH patients has dramatically changed the clinicians' management and patients' perspective toward this disease.

Reducing blood transfusion requirement and stabilizing Hb level

Symptoms of PNH are often disproportionate to the degree of anemia.[11] However, achieving sustained stability of Hb and transfusion independence were the main outcomes measured with eculizumab therapy in PNH studies.[11–13] Those two outcomes were chosen because both are measurable, and can be easily compared with pre- or no-eculizumab treatment end points to assess its response. Studies assessing the transfusion requirement and transfusion independence rates with eculizumab are illustrated in Table 1.

In the TRIUMPH study, 43 patients on eculizumab were compared with 44 patients on placebo in a double-blind randomized controlled trial, with the outcomes being the stabilization of Hb and transfusion independence.[11] Hb stabilization occurred in 49% in the eculizumab group compared to 0% in the placebo group ($P<0.001$). Furthermore, there was a significant reduction in the median number of units transfused in eculizumab group as compared to the placebo group. Similar findings were also reported in the SHEPHERD study.[13]

Kelly et al evaluated 79 consecutive PNH patients over 8 years, and compared them with 30 matched historical

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Table 1 Studies assessing hematological response and transfusion requirement in PNH patients treated with eculizumab

Study	Total N	Hb stabilization	Median number of PRBC units transfused (eculizumab vs placebo)	*P*-value	Overall transfusion reduction	% of transfusion independence	Transfusion reduction in dependent group
TRIUMPH[11]	87	49% ($P<0.001$)	0 vs 10	<0.001	73%	51% (at 6 months)	Yes
SHEPHERD[13]	96	–	0 vs 8	<0.001	52%	51% (at 12 months)	–
Kelly et al[8]	79	–	5 vs 19 (mean number)	<0.001	–	66% (at 6 months)	Yes ($P<0.03$)
Hillmen et al[17]	195	–	–	–	54.7%	82% (at the last 6 months of study)	Yes ($P<0.001$)
AEGIS[18]	29	31% Hb increase ($P<0.001$)	0.7 vs 4.9	0.0016	–	96% (at 18–24 months)	–

Abbreviations: PNH, paroxysmal nocturnal hemoglobinuria; Hb, hemoglobin; PRBC, packed red blood cell.

controls from the pre-eculizumab era.[8] The 12-month mean transfusion requirement was significantly reduced in patients receiving eculizumab compared to controls. Although this study was non-randomized, the inclusion of consecutive patients might reduce the risk of bias.

Long-term safety and efficacy data of eculizumab have been reported in two studies: the study by Hillmen et al and AEGIS study.[17,18] The first study included a total of 195 patients from three Phase III trials with a maximum duration of 66 months, and reported a total transfusion independence in 82% of patients.[17] The AEGIS trial was an open-label, noncomparative, multicenter Phase II study of 29 Japanese PNH patients, followed by a further 2-year extension study.[18] Only 27 patients were included in the extension study with a total transfusion independence of 96%.[17,18] The importance of the AEGIS extension study lies in the fact that it is the first study reporting long-term data of eculizumab in a trial setting among Asian PNH patients, a population in which PNH seems to be more frequent than in the Western population.[19]

In patients in whom transfusion independence was not reached, the TRIUMPH trial[11] and the studies by Kelly et al[8] and Hillmen et al[17] reported similar findings of reduction in the number of red cell units transfused after starting eculizumab as compared to before eculizumab treatment.

Finally, a recent retrospective study included 123 PNH patients treated with eculizumab and 191 historical controls from the French registry. Although the transfusion requirements were not reported in the study, the authors did suggest that anemia could persist in some patients.[1]

Improving QoL

QoL is affected in the majority of patients with PNH with 96% complaining of fatigue and >40% having symptoms of smooth muscle spasm often of sufficient severity to require narcotic analgesics.[20] Fatigue is multifactorial and is not simply the result of anemia, as 60% of these patients are transfusion independent.[20] The fluctuation in Hb and the inconvenience of the frequent blood transfusions may also contribute to the impaired QoL. Nevertheless, eculizumab has impacted favorably on the QoL in both transfusion-dependent and transfusion-independent patients.[3,9,11,21,22]

In the TRIUMPH study, QoL assessments were performed with the use of two instruments: the Functional Assessment of Chronic Illness Therapy – Fatigue (FACIT-F) scale and the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire. Patients in the eculizumab group had a mean increase (improvement) in scores on the FACIT-F of 6.4±1.2 points from baseline to week 26, compared to the placebo group in which the mean score decreased by 4.0±1.7 points during this period ($P<0.001$).[11] Importantly, only transfusion-dependent patients were included, and QoL was assessed prior to transfusion and after eculizumab, without posttransfusion assessments. Therefore, it is not possible to fully determine whether differences are a result of eculizumab or the anemia correction.[2] However, improvement in the FACIT-F score was also noted independent of improvement in Hb level in patients receiving eculizumab as compared to placebo, supporting the positive impact of eculizumab on QoL regardless of Hb level.

With respect to the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire, the eculizumab group had overall significant improvements in scores of functional status, fatigue, pain, dyspnea, loss of appetite, and insomnia, compared to placebo.[11] Similarly, improvements were demonstrated in several QoL parameters including physical, emotional, and cognitive functioning, global health, fatigue, dyspnea, and insomnia.[12] Similarly, the AEGIS study also showed a consistent improvement in QoL in the majority of patients with sustained reductions in fatigue and dyspnea.[18]

Of note, ~25% of PNH patients treated with eculizumab still need recurrent blood transfusions; however, even these patients report improved QoL.[3] Improvements in symptoms including abdominal pain, dysphagia, and erectile

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dysfunction have been reported during eculizumab therapy despite the observation that total Hb levels were unchanged or not completely corrected.[11,12,21] This supports the notion that intravascular hemolysis plays an important role in diminishing QoL in PNH patients.[11]

Reducing intravascular hemolysis

As previously mentioned, intravascular hemolysis results in symptoms of smooth muscle dystonia, including abdominal pain, dysphagia, and erectile dysfunction.[3,11] Eculizumab therapy results in a reduction of hemolysis and a new steady-state Hb level, due to balance of the underlying bone marrow dysfunction, the increased half-life of PNH erythrocytes, and the new level of transfusions (if any) required.[11] High percentages of PNH-type red blood cells have been observed after eculizumab treatment, indicating that these cells have not undergone hemolytic destruction.[10,18]

Lactate dehydrogenase (LDH), a biochemical marker of hemolysis, is used frequently to assess intravascular hemolytic activity. In the pre-eculizumab era, PNH patients with hemolysis (LDH $\geq$1.5$\times$ upper limit of normal) had a 4.8-fold increase in mortality compared to the general population.[23] The TRIUMPH study showed a rapid, significant, and sustained reduction in the LDH to normal or near-normal levels in patients on eculizumab.[11] Other studies have reported similar findings that last beyond 8 years of eculizumab therapy, reflecting inhibition of chronic hemolysis.[8,17,18]

Preventing thrombosis

Thromboembolism occurs in 50% of patients, and represents the major cause of death in PNH.[6,24,25] Once an initial thrombosis occurs, the risk of further thrombotic events increases, with a five- to ten-fold increase in mortality.[8] Interestingly, TE events in PNH are not restricted to the venous system.[4,6,22] Eculizumab was able to show a substantial impact in lowering the risk of thrombosis in PNH as summarized in Table 2.

It has been shown that patients on eculizumab have a significant and sustained decrease in activation of both the plasmatic hemostatic system and the endothelium.[26] Compared to pretreatment values, patients on eculizumab were found to have lower levels of coagulation factors such as von Willebrand factor, of by-products of coagulation such as D-dimer, and also of endothelial cell molecules such as vascular cell adhesion molecule 1.[26] These changes might be due to the blocking effect of eculizumab on C5, thus reducing platelet and endothelial activation, intravascular hemolysis, and release of thromboplastin-like substances.[7,27]

Table 2 Studies assessing thromboembolism rates with eculizumab

Study	TE event rate/100 PYs with eculizumab vs without eculizumab	*P*-value	Notes
Hillmen et al[22]	0.72 vs 11.54	<0.001	
Kelly et al[9]	0.8 vs 6.5	<0.001	
AEGIS[18]	Zero TE events		
Hillmen et al[17]	3.6% vs 32.6%[a] 2.3 vs 7.3[b]		96.4% of total patients free of thromboembolism
Loschi et al[1]	4% vs 29%[a]		

Notes: [a]Percentage, not rate. [b]Incidence rate calculated from information available in the study.
Abbreviations: TE, thromboembolic; PYs, person-years.

Initial studies were not designed to establish the effect of eculizumab on thrombosis as most trial patients were receiving concomitant anticoagulants.[2,12,13] However, in later studies, eculizumab has consistently shown a protective effect against thrombosis.[8,17,22,26] In a prespecified analysis, data from three combined studies[11–13] showed that TE rate in patients receiving exclusively anticoagulants was reduced from 11.54 events per 100 patient-years before eculizumab treatment to 0.72 events per 100 patient-years during eculizumab treatment ($P<0.001$), with a relative risk reduction of 94%.[22] Despite heterogeneity in the individual pre-study TE event rates, eculizumab treatment consistently resulted in a marked reduction in thromboembolism (Table 2). Other studies have also reported dramatic reductions in thrombosis after initiating eculizumab therapy (0.8 vs 5.6 events per 100 patient-years; $P<0.001$) even after accounting for the shorter duration of follow-up since starting eculizumab.[8] Of note, in the latter study, 50% of the pre-eculizumab thrombosis events occurred on anticoagulants, which supports the notion that in PNH patients with a previous history of thrombosis, anticoagulation alone may not be sufficient in preventing thromboses. On the other hand, after eculizumab treatment, primary prophylaxis with warfarin was stopped, whereas anticoagulation for secondary prophylaxis was not routinely stopped. Thus, cessation of anticoagulation in patients on eculizumab with no prior history of thrombosis is the current recommended practice in some centers.[8] Similar TE reductions have been reported in other studies.[17,18] TE events decreased by 81.8%, with 96.4% of patients remaining free of TE events.[17] In the AEGIS study, no TE events were reported during the 2-year extension period.[18]

A recent study from the French registry also provided evidence for significantly lower TE rates in patients on eculizumab compared to historical controls (4% vs 29%).

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However, the study showed that thromboembolism remains a major issue in PNH, even in the eculizumab era, as highlighted by the negative impact of a previous thromboembolism on OS in adjusted analysis (hazard ratio [HR] 4.48, 95% confidence interval [CI] 2.46–8.16; $P<0.001$).[1]

Improving renal function

Renal dysfunction has been well reported in PNH,[11,18] and patients with PNH have a sixfold greater risk of chronic kidney disease (CKD) compared to the general population.[9,11,18] More importantly, CKD is associated with an elevated risk of premature mortality contributing to 8%–18% of PNH-related deaths.[6,17] Renal impairment in PNH is multifactorial and involves a direct effect of nitric oxide depletion, induced by cell-free Hb, on the glomerular filtration rate and on renal plasma flow, and the repetitive exposure to cell-free Hb leading to chronic hemosiderin deposition in the proximal renal tubules.[9,10] Supportive care measures do not prevent the occurrence or the progression of CKD. However, by inhibiting terminal complement activation and intravascular hemolysis, treatment with eculizumab significantly improves renal function. Several studies have shown consistently that eculizumab has a protective role leading to improvement in, or less progression of, renal disease.[9,10,17]

Hillmen et al provided evidence that sustained long-term therapy with eculizumab can reverse early-stage renal damage sustained prior to treatment initiation, particularly when administered early and prior to advanced renal impairment.[17] In this study, CKD stages[28] were assessed following each 6-month eculizumab treatment period. Continued time-dependent improvement in CKD was observed with sustained eculizumab treatment, with 24.4% of patients showing improvement in CKD after only 6 months of treatment and 45% of patients showing improvement after 36 months. Overall, patients were significantly more likely to experience an improvement than a worsening in renal function ($P=0.015$).[17]

Similar findings have been reported in the AEGIS study. Sustained administration of eculizumab in patients with CKD at baseline was associated with improved renal function in 71%, whereas 29% had their renal function stabilized. Eculizumab also improved renal function, particularly if administered early in the course of disease and before the kidney is more severely impaired. Improvements in renal function were observed in 88% of patients with stage 1 CKD, 67% of patients with stage 2 CKD, and 33% of patients with stages 3–4 CKD.[18]

A large cohort of 195 PNH patients from parent eculizumab clinical trials was also assessed for their kidney function by Hillmen et al.[9] In this study, improvement in renal function was more commonly seen in patients with baseline CKD stages 1–2 (67.1% improvement), although improvement was also observed in patients with CKD stages 3–4. Overall, 40 (21%) of 195 patients who demonstrated renal dysfunction or damage at baseline were no longer classified as such after 18 months of treatment.[9]

Improving OS

Historical studies have shown the median survival in PNH patients to be between 10 and 15 years from the time of diagnosis.[4] More recently, data from a French series of patients in the pre-eculizumab era showed an improvement in median survival to 22 years, which is probably the result of improvements in supportive care and in the management of disease complications.[25] With the use of eculizumab, the OS of PNH patients has consistently shown a remarkable improvement, approaching that of the general population.[1,8,17] The survival rate of PNH patients in eculizumab studies was 97.6% at 3 years,[17] 95.5% at 5 years ($P<0.03$ as compared to eculizumab-untreated patients),[8] and 92% at 6 years ($P=0.037$ as compared with controls).[1,8,17] Furthermore, Kelly et al reported no significant difference in OS between the PNH patients treated with eculizumab compared with sex- and age-matched controls from the UK population.[8] Another study suggested that the two factors statistically associated with a worse OS were the absence of treatment with eculizumab (HR 6.26, 95% CI 2.72–14.43; $P<0.001$) and a previous history of TE events (HR 4.48, 95% CI 2.46–8.16; $P<0.001$).[1]

Absent or inadequate response to eculizumab in PNH

It has been reported that, with eculizumab, up to two-thirds of PNH patients who are transfusion dependent become transfusion independent.[3] However, ~25% of them still need recurrent albeit less frequent blood transfusion.[3] The authors suggested that these heterogeneous hematological responses may be related to factors including underlying AA,[1–3] C3-mediated extravascular hemolysis,[3,15,29] breakthrough hemolysis,[30] or the presence of a specific mutation in the C5 gene which prevents eculizumab from binding to C5 protein.[6]

The association of PNH with AA

In some patients, a poor or suboptimal response to eculizumab may indicate that the extent to which bone marrow

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failure was contributing to anemia had been underestimated.[3] There is a close relationship between AA and PNH, and a history of severe AA is present in at least one-half of all newly diagnosed PNH cases,[31] whereas PNH clones are found in up to 70% of patients with AA.[2,31] Recently, new evidence on PNH pathogenesis supports the notion that expansion of the PNH clone results from escape from T-cell-mediated autoimmune damage to normal hematopoietic stem cells, with the GPI molecule as target, consolidating further the observed association between PNH and AA. Indeed, GPI-specific $CD8^+$ T cells – which have been identified in PNH patients – would spare selectively GPI-negative stem cells, thus enabling them to repopulate the marrow of a patient who would otherwise have AA.[3]

In the AEGIS trial, the effect of eculizumab was assessed in patients with PNH plus AA/myelodysplastic syndrome (MDS) and those with PNH alone, and no significant differences were found between the two groups in the end points assessed, namely hemolysis and blood transfusion. This finding suggests that patients with PNH and AA/MDS experience similar hemolysis-associated complications and can benefit from treatment with eculizumab.[18]

Another interesting observation has been reported by a case report of a PNH patient who was initially diagnosed with AA and treated subsequently with eculizumab.[32] Erythropoiesis improved, and the patient became transfusion independent shortly after initiation of iron chelation with deferasirox.[32] Similarly, the same finding has also been seen in MDS patients after starting deferasirox.[33,34] The significance of this management modality needs to be addressed in future studies.

It is too early to draw robust conclusions on the effect of eculizumab on patients with PNH plus AA, yet the evidence of the association between the two suggests that an inadequate marrow response to anemia may be a contributing factor to the observed persistent anemia in PNH patients receiving eculizumab. More importantly as many as 25% of patients with a previous diagnosis of AA treated with immunosuppression may progress on to develop hemolytic PNH requiring therapy.[35]

Extravascular hemolysis with eculizumab

Two studies have described the development of "iatrogenic" extravascular hemolysis with eculizumab.[15,29] Risitano et al have suggested that binding of C3 by PNH red blood cells may constitute an additional disease mechanism, strongly enhanced by eculizumab treatment and producing a variable degree of extravascular hemolysis.[15] Eculizumab affects only the distal complement pathway, thus neutralizing the deficiency of CD59 on PNH red blood cells by inhibition of C5 cleavage, but since these are also deficient in CD55, upstream complement activation may lead to C3 opsonization leading to a gradual accumulation of C3 fragments on their surface. These C3-opsonized red blood cells will be susceptible to phagocytosis by macrophages in the reticuloendothelial system, resulting in extravascular hemolysis.[3,7] This phenomenon may explain the residual low-level hemolysis occurring in some eculizumab-treated patients,[29] indicated by a positive Coombs test (classically negative in untreated PNH).[3,7,29]

More importantly, researchers have suggested that this process may play a role in limiting anemia improvement because it develops at the same time that intravascular hemolysis ceases.[3,7] However, fortunately in many cases, it seems to have little or no clinical relevance.[7] This conclusion has also been supported by studies in which a dramatic reduction in transfusion requirements was observed, indicating that the phenomenon of extravascular hemolysis is of limited clinical importance for most patients.[8,17] In the next decade, researchers may quest for a way to control extravascular hemolysis.[7] It has been recommended that selective splenic artery embolization can be used to treat severe extravascular hemolysis, as an alternative to splenectomy.[3] Of note, selective splenic artery embolization has also been used to reduce spleen size and alleviate cytopenias in splenomegaly with hypersplenism in PNH patients.[3]

Breakthrough hemolysis

According to Nakayama et al, eculizumab-dosing intervals >17 days may be associated with an increased risk of developing breakthrough hemolysis in patients with PNH (odds ratio 1.6, 95% CI 1.3–2.0; $P<0.01$).[30] The recommended schedule is thus to maintain eculizumab administration every 14 days, although this needs to be further confirmed.[1,2] Other factors may also account for breakthrough hemolysis such as pregnancy (detailed in the "Eculizumab in pregnancy" section).

C5 gene mutation

C5 gene mutation has been detected among Japanese patients with PNH, identified as having poor response to eculizumab.[6] A poor response was defined as markedly high levels of LDH during treatment, indicating that intravascular hemolysis remained unaffected, regardless of the persistence or improvement in other laboratory findings or clinical symptoms. This mutation prevents binding and blockade

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by eculizumab while retaining the functional capacity of the mutant C5 to cause hemolysis. The prevalence of this mutation among the patients with PNH (3.2%) was similar to that among healthy Japanese persons (3.5%).

Special considerations

Meningococcal meningitis and other infections

Complement inhibition by eculizumab leads to impairment in neutrophil and monocyte function, consequently affecting their ability to clear infections by encapsulated organisms, such as *Neisseria meningitidis*.[2,8] Patients who are to be treated with eculizumab must be vaccinated against *N. meningitidis* at least 2 weeks prior to starting treatment.[2]

The tetravalent meningococcal vaccine is available against subgroups A, C, W, and Y but not for serogroup B, which is the most common serotype isolated in meningococcal sepsis in areas such as the UK.[8,9] This explains the reported rates of meningococcal infections in some studies despite all patients being vaccinated appropriately.[8,9] Hillmen et al and Kelly et al reported a rate of 1% and 2.5%, respectively, for meningococcal meningitis on eculizumab.[8,9] Therefore, in January 2010, the UK policy changed to include antibiotic prophylaxis for all patients (penicillin V 500 mg twice a day or erythromycin 500 mg twice a day for patients intolerant to penicillin), in addition to the tetravalent meningococcal vaccination.[8] In 2013, Bexsero® became the first broad-coverage vaccine to be licensed for active immunization against serotype B meningococcal disease.[36] Bexsero is now licensed in >35 countries worldwide for varying age groups, including the European Union, Australia, Brazil, Canada, Chile, Uruguay, and the USA, and it is usually combined with the tetravalent vaccine in PNH patients planned for eculizumab for full coverage of the five serotypes.[36] However, in daily practice, clinicians may still prefer to continue on penicillin or amoxicillin prophylaxis while awaiting the vaccines to kick in, and some would still choose to continue penicillin even after vaccinated because other subtypes may not be covered.

Other infections have also been reported in eculizumab studies. The AEGIS study reported a total of 159 treatment-emergent infections, of which the majority were mild in severity (87.4%). Only seven of the 159 infections (4%) were considered probably related to eculizumab, of which three (one case each of pneumonia, cellulitis, and sepsis) were severe. There were no meningococcal infections reported.[18] Hillmen et al also reported an overall infection rate of 12.8%.[9]

Overall, studies indicate that eculizumab is well tolerated over the long term, and its profile is consistently safe across studies. However, further precautions might be considered in immune-compromised categories such as elderly or neutropenic patients.

Eculizumab in pregnancy

The median age at diagnosis of PNH is 34 years, so affected females are often in the reproductive age group.[37] Although PNH is not known to adversely affect female fertility, several fetal–maternal complications have been reported in pregnant PNH patients related to exacerbated intravascular hemolysis, including spontaneous miscarriages (45%), premature births, increased blood transfusion requirements, thrombocytopenia, thromboembolism, and accelerated preeclampsia.[37–39] In PNH, maternal mortality has been reported to range between 8% and 21%, mostly occurring in the postpartum period, and thromboembolism being the primary cause. On the other hand, fetal mortality has been reported to range between 4% and 9%, mostly associated with premature births.[38,40,41] Close monitoring of all pregnant patients with PNH is thus recommended.

Pregnancy activates the complement cascade, with increased terminal complex formation in the third trimester.[14,42] Therefore, PNH has been considered a relative contraindication for pregnancy, and patients were usually discouraged from conceiving.[37,39] Because of the risk of life-threatening thrombotic complications, pregnant women with PNH in the pre-eculizumab era were to receive full anticoagulation during pregnancy and for at least 4–6 weeks postpartum.[38]

Several studies and case reports have described the use of eculizumab in pregnant PNH patients.[32,37,39,43–45] It has been noted that the dose of eculizumab required to block complement during the later stages of pregnancy is greater than that required in the nonpregnant state.[44] The increased activity of lysosomal enzyme, as well as the physiologic changes that occur during pregnancy, such as the physiologic expansion of intravascular volume, may account for the higher doses required.[32,39]

In pregnant patients, the eculizumab schedule has been modified by reducing the interval between eculizumab dose administration to ~7–12 days,[43–45] by administering higher doses of eculizumab (1,200 mg every 2 weeks during the third trimester),[37] or by using both modalities together,[32,39] according to physician discretion in order to control hemolysis as pregnancy progresses.[32,37,39,43–45]

The largest PNH study in pregnancy so far has been reported by Kelly et al in 2015.[39] Data were reported on 75 pregnancies in 61 women with PNH from the International PNH Interest Group and the International PNH Registry.[39] In 54% of

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pregnancies that progressed past the first trimester, the dose or the frequency of use of eculizumab had to be increased. Requirements for transfusion of red blood cells increased during pregnancy, from a mean of 0.14 units per month in the 6 months before pregnancy to 0.92 units per month during pregnancy, and platelet transfusions were required in 21% of pregnancies, mostly administered at the time of delivery.[39] Furthermore, premature birth rate of 29% was reported. Two TE events were documented, and both occurred during the postpartum period, despite that thromboprophylaxis with low-molecular weight heparin was used in the majority of patients (88%). One of the thrombotic events occurred after a plasma infusion was administered to treat a postpartum hemorrhage. Plasma contains high levels of complement and can overcome the effects of eculizumab and thereby render the patient susceptible to complications of PNH. The use of plasma should probably be avoided if possible.

Initially, it was thought that eculizumab would cross the placenta and appear in breast milk.[2] However, subsequent studies reported that eculizumab does not appear to cross the placenta nor it is excreted in breast milk.[37,43–45] However, in a study on 21 cord-blood samples, eculizumab was detected in seven of the samples, whereas the drug was not detected in any of the ten breast milk samples tested.[39] Recently, two studies suggested that if eculizumab crosses the placenta, the level is not high enough to affect complement activation.[39,46]

Overall, studies highlighted the significance of regular and frequent monitoring of hemolytic parameters in PNH patients during pregnancy to detect early disease activity. In the end, there are no adequate and well-controlled studies of eculizumab in pregnant women, and a firm conclusion is difficult to be drawn. Nevertheless, all studies available suggested that eculizumab provided benefit for women with PNH during pregnancy, as evidenced by a high rate of fetal survival, a low rate of fetomaternal complications, and a reduced rate of blood transfusion, compared to pregnant PNH patients not treated with eculizumab.[37,39,43–45] It also appears that eculizumab can be safely used throughout pregnancy.

In summary, the recommended practice in pregnant women with PNH receiving eculizumab includes dose escalation and/or reduction in dose interval to control hemolysis, avoidance of the use of plasma if possible, and thromboprophylaxis with low-molecular weight heparin throughout the pregnancy up to 6 weeks following delivery, even with the use of eculizumab. However, whether combining thromboprophylaxis with eculizumab (specifically during the postpartum period) would be sufficient for preventing thromboembolism, or whether dose escalation is needed in high-risk patients, such as those having a prior history of thromboembolism or requiring C-section, still remain as questions.

Eculizumab in elderly

The median age at diagnosis of PNH is in the early 30s, and thus, patients aged >80 years were rarely enrolled, yet PNH can still present at any age.[5,6,37] In view of the known age-associated reduction in immune function and the effect of eculizumab on the suppression of the terminal complement pathway, one concern is that treatment with eculizumab may elevate the risk of infection in elderly patients.[10] In the TRIUMPH and SHEPHERD trials, there were no apparent age-related differences observed in patients aged 65 years or older treated with eculizumab. However, the number of those patients was only 15, and clearly, this number would not be sufficient to determine whether they respond differently than younger patients.[2]

A case report by Ooe and Nagai in Japan has described the use of eculizumab in an 86-year-old PNH patient.[10] Despite that poor prognosis was considered in this patient due to PNH-induced chronic renal failure and concomitant underlying myelodysplasia, eculizumab use was associated with rapid suppression of hemolysis, and significant improvement in both blood transfusion requirement and renal function from CKD stage 4 to stage 3 with no infections reported.

Although findings from available studies suggest that eculizumab can be used safely in elderly PNH patients with proved efficacy, the overall experience of eculizumab in geriatric population is still very limited, and decisions should be made on a case-by-case basis.

Risk of MDS/acute leukemia with eculizumab

An important concern with eculizumab is the possibility of clonal evolutions arising from a prolonged complement blockade. One study reported no significant difference regarding clonal evolution to MDS or acute myeloid leukemia in both eculizumab and control cohorts.[1] However, no conclusions can be drawn, since a longer follow-up will be necessary as the median time to clonal evolution is 10 years.[1] Furthermore, the rate of clonal myeloid evolution into MDS or acute leukemia was reported to be ~4% in PNH patients receiving eculizumab in another study,[8] which is a similar rate reported in PNH patients before eculizumab therapy.[6,25,26] Interestingly, in the study by Kelly et al, MDS and/or acute leukemia occurred within the GPI-negative clone of cells, indicating that the majority of hematopoiesis in these patients

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was derived from PNH stem cells.[8] Long-term administration of eculizumab to Japanese patients with PNH showed that the burden of PNH disease and baseline characteristics were similar in patients with and without a history of bone marrow dysfunction such as AA or MDS.[18] No cases of clonal evolution were reported by Hillmen et al.[17]

In fact, the effect of eculizumab on the development of clonal diseases is still unclear, and long-term data are definitely required.

Paucity of long-term "real-world" data

In view of the rarity of PNH, and the relatively recent introduction and approval of eculizumab, long-term data are scarce. Perhaps, the best standardized long-term safety and efficacy data of eculizumab were presented by Hillmen et al.[17] They investigated 195 patients with PNH who had participated in one of three prospective parent trials: the Phase II pilot study and its extensions – TRIUMPH and SHEPHERD trials.[11–13] The median eculizumab treatment duration was 30.3 months, with a maximum duration of 66 months. Overall, long-term treatment with eculizumab resulted in sustained improvement in patient outcomes by rapidly reducing hemolysis and improving patient survival due to significant reduction in the frequency of severe and life-threatening morbidities, such as TE events and CKD with a favorable safety profile. However, "real-world" data with long-term follow-up are still lacking. The International PNH Registry (www.pnhregistry.com) is providing further opportunities to continue studying and understanding the long-term benefits of eculizumab in patients with PNH outside the clinical trial setting.[17]

Conclusion

The advent of eculizumab has resulted in a dramatic improvement of the natural history of PNH patients with improvement in virtually every aspect of these patients' lives. This is supported by the available data about which some limitations should be noted. First, the size of available studies is relatively small but dictated by the rarity of the disease. Second, most (if not all) research is funded by the pharmaceutical industry, which conveys an inherent risk of bias. Third, availability of randomized data is scarce, and many studies use historical controls, also risking the introduction of bias. Fourth, the treatment is very costly limiting access to it. In spite of these limitations, the data available so far are strong enough (and differences quite striking in some cases) to support the use of this therapy, although independent confirmatory data are sorely needed. Fifth, notwithstanding the available evidence, many questions remain unanswered, in particular regarding long-term safety (eg, infections, clonal evolution), optimal management of special populations (eg, pregnant, elderly), role of eculizumab in PNH/AA, and need/utility of clinical and laboratory monitoring, among others. Finally, since the main deterrent to a wide adoption of this therapy is mainly of an economic nature and many patients who might benefit from it are unable to access this treatment, patient support programs and access to public funding as well as novel and more accessible agents are highly desirable.

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Antibody Framework Residues Affecting the Conformation of the Hypervariable Loops

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Rodent monoclonal antibodies have been "humanized" or "reshaped" for therapy by transplanting the antigen-binding loops from their variable domains onto the β-sheet framework regions of human antibodies. However, additional substitutions in the human framework regions are sometimes required for high affinity antigen binding. Here we describe antigen binding by a reshaped antibody derived from the mouse anti-lysozyme antibody D1.3, and several variants in which point mutations had been introduced into framework positions to improve its affinity. The affinities were determined from the relaxation kinetics of reactant mixtures using quenching of fluorescence that occurs upon formation of the antibody–antigen complex. The dissociation constant of lysozyme ranged from 3·7 nM (for D1.3) to 260 nM. Measurement of antibody–antigen association kinetics using stopped-flow showed that D1.3 and most of the reshaped antibodies had bimolecular rate constants of $1{\cdot}4 \times 10^6$ s^{-1} M^{-1}, indicating that differences in equilibrium constant were predominantly due to different rates of dissociation of lysozyme from immune complexes. Mutations in a triad of heavy chain residues, 27, 29 and 71, contributed 0·9 kcal/mol in antigen binding free energy, and a Phe to Tyr substitution of light chain residue 71 contributed an additional 0·8 kcal/mol. The combined effect of all these mutations brought the affinity of the reshaped antibody to within a factor of 4 of D1.3. All of these substitutions were in the β-sheet framework closely underlying the complementarity-determining regions, and do not participate in a direct interaction with antigen. The informed selection of residues in such positions may prove essential for the success of loop transplants in antibodies. Variation of these sites may also have a role in shaping the diversity of structures found in the primary repertoire, and in affinity maturation.

Keywords: humanized antibody; kinetics; site-directed mutagenesis; hypervariable loop; lysozyme

1. Introduction

The antibody is a Y-shaped molecule, in which the variable (V) domains forming the tips of the arms bind to antigen and those forming the stem (C-domains) are responsible for triggering effector functions that eliminate antigen. X-ray diffraction studies of crystalline antibody fragments reveal the V and C-domains as two layers of β-pleated sheet, with loops connecting the ends of the β-strands (Poljak *et al.*, 1973; Schiffer *et al.*, 1973; Segal *et al.*, 1974). In the heavy and light chain V-domains the loops at one end of the sheet are hypervariable in sequence (Wu & Kabat, 1970; Kabat & Wu, 1971), and form the antigen-binding site. The β-sheet provides a scaffold for mounting a diversity of loops, and indeed the antigen binding site can be transplanted from a rodent antibody to a human antibody, thereby "humanizing" the rodent antibody, by transplanting these loops (Jones *et al.*, 1986; Verhoeyen *et al.*, 1988; Riechmann *et al.*, 1988). The clinical success of one such "reshaped" human anti-lymphocyte antibody, CAMPATH-1 (Riechmann *et al.*, 1988) in treating B-cell lymphoma (Hale *et al.*, 1988) and vasculitis (Mathieson *et al.*, 1990), has prompted the reshaping of antibodies directed against the interleukin-2 receptor (Queen *et al.*, 1989), CD4 (Gorman *et al.*, 1991) respiratory syncytial virus (Tempest *et al.*, 1991), herpes simplex virus (Co *et al.*, 1991), human immunodeficiency virus (Maeda *et al.*, 1991) and epidermal growth factor receptor (Kettleborough *et al.*, 1991).

However, reshaping requires that the rodent and human framework regions are structurally conserved, both in the orientation of the two β-sheets of each domain and in the packing of the heavy and light chain V-domains together; that the

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hypervariable loops make the majority of contacts with antigen; and that the loops are supported in a similar way by the underlying β-sheet framework. Although these are likely to be true for some antibodies, the restitution of key contacts between loops and framework has proved necessary in others, and has been assisted by molecular modelling (Riechmann *et al.*, 1988; Tempest *et al.*, 1991) and systematic matching of rodent and human framework regions to minimize differences in primary sequences (Queen *et al.*, 1989; Gorman *et al.*, 1991; Maeda *et al.*, 1991). As a model we have reshaped a human antibody based on the hypervariable regions of mouse antibody D1.3 (Amit *et al.*, 1986; Bhat *et al.*, 1990) and the framework regions of human Vκ(I) family (Kabat *et al.*, 1987) and the myeloma protein NEW (Poljak *et al.*, 1973). The parent antibody structures have all been solved crystallographically. The Bence–Jones protein REI is in the Vκ(I) family (Epp *et al.*, 1974), and the D1.3 antibody was solved alone and in complex with the antigen lysozyme (Amit *et al.*, 1986; Bhat *et al.*, 1990). We achieved large enhancement of antigen affinity by substitution of several framework residues of a group that may exert a determining influence on the conformation of the CDRs†.

2. Antibody Design

The original distinction between hypervariable regions and framework residues (Wu & Kabat, 1970) had its basis in homologies between the primary sequences of immunoglobulins known at that time. However, as X-ray crystallographic structures became available, it became apparent that residues in the hypervariable regions formed the apical loops connecting the β-strands of the immunoglobulin fold, but could also extend part way along the β-strands themselves (Poljak *et al.*, 1973; Schiffer *et al.*, 1973; Segal *et al.*, 1974). Indeed structural analyses have invoked fewer, and in some cases different, residue positions as CDRs (for example, see Chothia & Lesk, 1987) In X-ray crystallographic structures of antigen–antibody complexes, all the CDRs do not necessarily make contact to antigen (Tulip *et al.*, 1989) and in particular the C-terminal portion of VH-CDR2 has never been shown to interact directly with antigen.

We based our designs on Kabat (Kabat *et al.*, 1987). The construction of the reshaped heavy chain has been described (Verhoeyen *et al.*, 1988). The framework amino acid sequences chosen for the light chain CDR acceptor were designed *de novo*. They are a consensus of human κ subgroup I sequences (Kabat *et al.*, 1987) and closely related (but not identical) to the sequence of the myeloma protein REI (Palm & Hilschmann, 1973). The framework sequences of the first reshaped antibody differ from D1.3 at 18 positions in the light chain and 30 in the heavy chain. The location of these residues is shown in the α-carbon traces of the variable domains of the D1.3 structure in Figure 1(a). The differences are dispersed over the entire variable region of the antibody: all eight framework segments have multiple replacements. The most numerous, half of the total, occur in framework 3 (between CDR2 and CDR3) of both the light and heavy chain. As expected, few differences are seen at the VH–Vκ framework interface, as this region is generally conserved. Two-thirds of the framework differences are located on the molecule's surface and are unlikely to affect antigen binding. There are also differences in buried residues, particularly at the interfaces with the hypervariable regions. We considered retaining these buried framework residues from mouse D1.3 in the reshaped antibody (Padlan, 1991), but even buried residues can form the critical element of a T-cell epitope if presented as a denatured peptide by a class II MHC molecule (Allen *et al.*, 1985). This could exacerbate any humoral response to the native antibody, for example an anti-idiotype reaction, and we therefore used the entire human framework regions, whether the residues were buried or not.

To probe the role of specific framework residues in supporting the CDR conformations, we introduced several additional framework substitutions into the reshaped antibody (VL: Phe71 to Tyr; VH Ser27 to Phe, Thr28 Phe29 Ser30 to Ser Leu Thr, and Lys71 to Val; see Fig. 1(b)).

Residue 71 of the light chain lies in a loop connecting β-strands and the tyrosine ring of D1.3 protrudes inward and is sandwiched between this loop and VL-CDR1 loop. The phenolic oxygen appears to be important: it hydrogen-bonds to the amide nitrogen and carbonyl of Gly68 and the backbone amide nitrogen of Asn31, thus forming a bridge between the two loops. The neighbouring residue Tyr32 is in intimate contact with lysozyme residue Gln121, previously identified as a central feature of the D1.3 recognition site (Amit *et al.*, 1986). Residue Phe71 was therefore changed to Tyr.

Residues 27 to 30 of the heavy chain are part of a structural loop which includes VH-CDR1 (Kabat *et al.*, 1987). Indeed they are included within a structure-based hypervariable loop that comprises residues 26 to 32 (Chothia & Lesk, 1987) compared with VH-CDR1 that comprises 31 to 35 (Kabat *et al.*, 1987). These residues appear to make important interactions with CDR1 and CDR2. For example, in the D1.3 structure, Leu29 packs against Phe27 and Lys71 (see below), and Phe27 packs against residues 31 to 34. In turn Gly31 and Tyr32 of VH-CDR1 make contact with Lys16 of lysozyme. Residue Ser27 would be expected to create a cavity, and was therefore changed to Phe as in D1.3 and Riechman *et al.* (1988). Residues Thr28, Phe 29, Ser30 were changed *en bloc* to Ser, Leu, Thr.

Residue 71 of the heavy chain may fix the relative dispositions of CDR1 and CDR2, according to

† Abbreviations used: CDR, complementarity-determining region; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; K_{eq}, equilibrium dissociation constant; kb, 10^3 bases or base-pairs.

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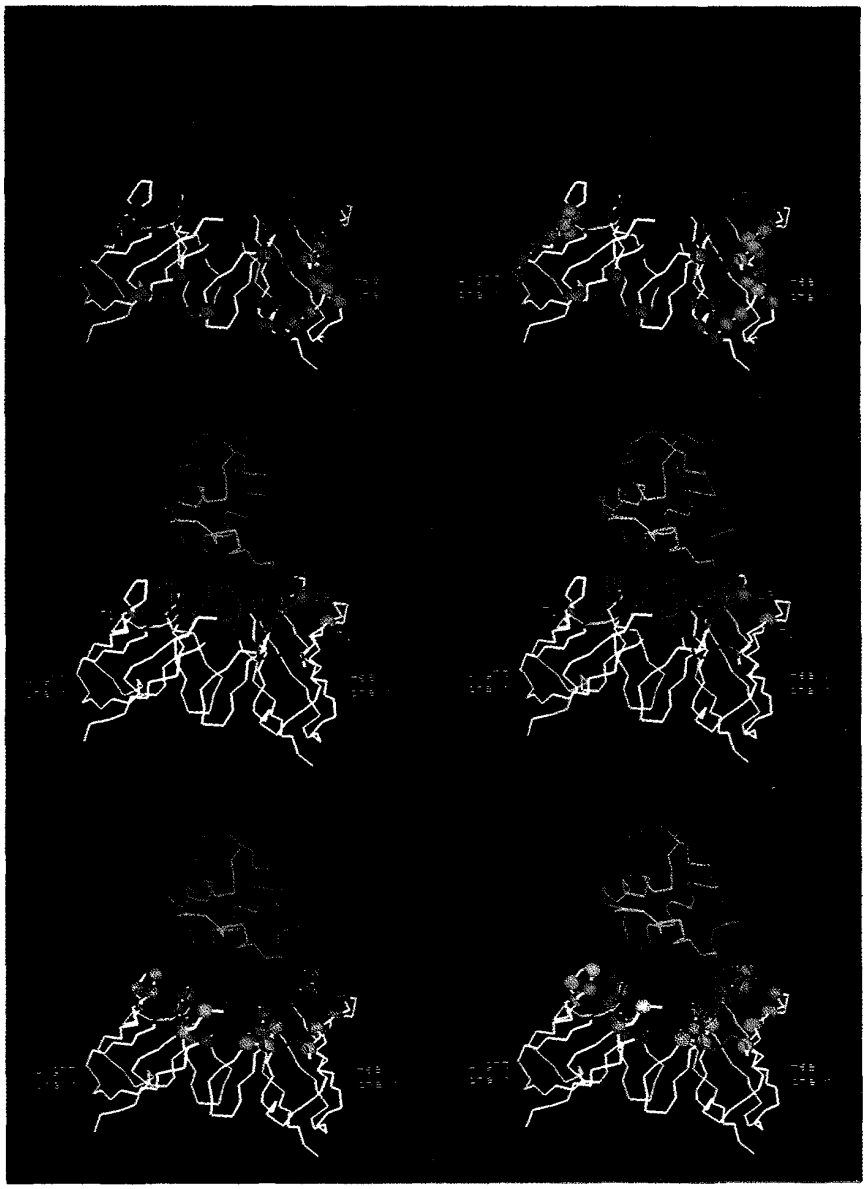


Figure 1. FabD1.3 complex with lysozyme. The α-carbon trace of the FabD1.3 (Fischmann *et al.*, 1991) is marked in white (framework region) and red (CDRs), and the α-carbon trace of lysozyme in blue. Residues corresponding to (a) differences between framework residues of mouse (D1.3) and reshaped antibody, (b) point mutations introduced into the framework of the reshaped antibody and (c) the Vernier zone, are highlighted in green.

whether there is bulky side-chain (Lys or Arg), or a smaller side-chain (Val, Ala) present (Tramontano *et al.*, 1990). We therefore changed Val71 to Lys as in D1.3.

3. Methods

(a) *Construction of reshaped light chain variable gene and Phe71 → Tyr mutant*

A myeloma expression vector used to produce engineered immunoglobulin heavy chains (Neuberger, 1983; Neuberger *et al.*, 1985) was adapted for light chain expression. This entailed synthesis and cloning the reshaped light chain V-gene and its introduction into the vector M13-HuVNP (Jones *et al.*, 1986) to replace the heavy chain variable gene.

A set of oligonucleotides was designed to encode the reshaped light chain V-gene with codon usage of mouse immunoglobulin sequences. However, the sequence encoding residues beyond number 96 of the mature protein was taken directly from the human J1 segment (Hieter *et al.*, 1982) including 30 nucleotides 3′ to the

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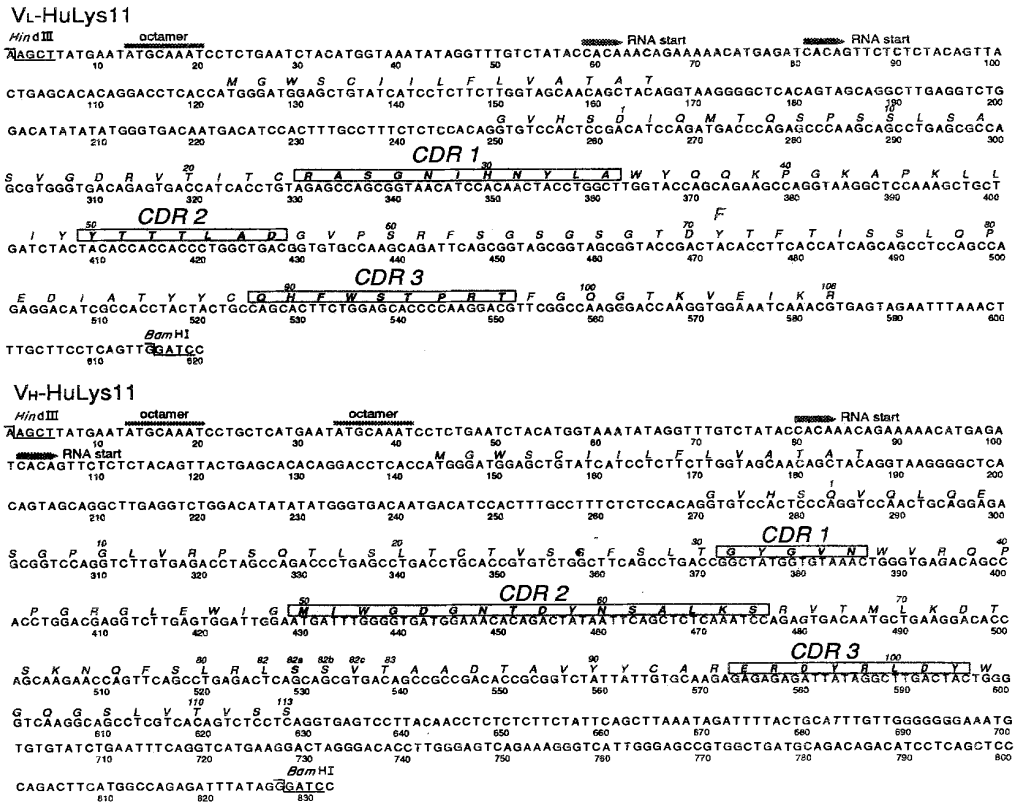


Figure 2. Nucleotide and encoded protein sequences of reshaped heavy and light chain variable domains. The DNA sequence encoding the highest-affinity reshaped antibody (HuLys11) is shown. The location of the mutations, transcriptional control motifs and protein translations appear above the corresponding nucleotide sequence, with nucleotide numbering underneath. Peptide numbering begins from the N-terminal residue of the mature protein. CDR sequences are boxed.

splice junction. Eighteen oligonucleotides were used to encode both strands of the 370 base-pair construct, and were assembled and cloned in three separate blocks, delimited by *Pst*I–*Kpn*I, *Kpn*I–*Kpn*I, and *Kpn*I–*Eco*RI sites. 50 pmol of each oligonucleotide was phosphorylated for 30 min at 37°C in a 20 μl reaction mixture with 1 mM-ATP, 5 units polynucleotide kinase, 5 mM-dithiothreitol, 50 mM-Tris, 10 mM-$MgCl_2$ (pH 8). Portions (4 μl) of each phosphorylation mixture were annealed by heating together at 80°C for 5 min, 67°C for 30 min, and cooling gradually (30 min) to room temperature. 200 nl of annealing mix was ligated into 20 ng M13-mp19 vector (Yanisch-Perron *et al.*, 1985) in a volume of 10 μl (composition as above except for 120 units of T4 DNA ligase and a pH of 7·5). Recombinant clones were sequenced and the 3 blocks excised, gel-purified and assembled by ligation into M13-mp19 as a *Pst*I–*Eco*RI fragment.

The synthetic gene was introduced as a *Pst*I–*Bam*HI fragment into the vector M13-pHVNP and joined in-frame to the signal sequence with a mutagenic oligonucleotide (5′-TCA TCT GGA TGT CGG AGT GGA CAC CT-3′) (Zoller & Smith, 1982). Further derivatives were made: about 1·2 kb of sequence 5′ to the immunoglobulin octamer transcriptional control element (Falkner & Zachau, 1984) was deleted using the oligonucleotide 5′-TAG ATT CAG AGG ATT TGC ATA TTC ATA AGC TTG GGC TAA TCA T-3′, and a point mutation, Phe71 to Tyr, was introduced using the oligonucleotide 5′-GGT GAA GGT GTA GTC GGT ACC-3′. The annotated sequence of the mutated gene is given in Fig. 2.

(b) *Construction of mutants of reshaped heavy chain variable gene*

Mutants of the reshaped heavy chain variable gene (Verhoeyen *et al.*, 1988) were constructed by oligonucleotide-directed mutagenesis. As above, 1·2 kb of sequence 5′ to the octamer motif was deleted, but here it also resulted in spurious duplication of the motif. Residues 27 to 30, initially Ser-Thr-Phe-Ser, were changed to Phe-Ser-Leu-Thr with the mutagenic oligonucleotide 5′-TAC ACC ATA GCC GGT TAA GCT GAA GCC AGA CAC GGT-3′, Ser27 to Phe with the oligonucleotide 5′-GCT GAA GGT GAA GCC AGA CAC G-3′, and Val71 to Lys with the oligonucleotide 5′-TGC TGG TGT CCT TCA GCA TTG TC-3′. The annotated sequence of the mutated gene is shown in Fig. 2.

(c) *Eukaryotic expression vectors*

Fragments carrying heavy and light chain variable genes with signal sequences and promoters (as above)

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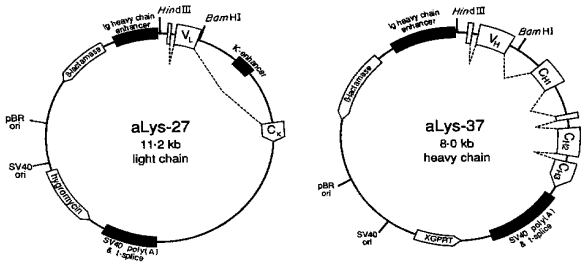


Figure 3. Vectors for expression of reshaped heavy (aLys37) and light (aLys27) chain variable regions as human $\gamma 1$ and κ chains in myeloma cells. Immunoglobulin exons are indicated by wide boxes, the coding regions of β-lactamase, aminoglycoside phosphotransferase (hygromycin resistance) and bacterial xanthine-guanine phosphoribosyl transferase by narrow boxes, and splicing between immunoglobulin exons by broken lines.

were now introduced into expression vectors. A vector pSV-$V_{NP}H\varepsilon$ (Neuberger *et al.*, 1985) based on the vector pSV2gpt (Mulligan & Berg, 1980), was further modified to facilitate rapid cloning of the variable genes and their co-expression as heavy and light chains in cultured myeloma cells. A human κ constant gene was built into a vector utilizing hygromycin resistance as a selectable marker, and a human heavy chain $\gamma 1$ gene built into a vector utilizing mycophenolic acid resistance (Fig. 3).

The assembly of the vector for light chain expression involved a ligation of 3 fragments: a 2 kb *Hin*dIII–*Bam*HI fragment containing the synthetic variable domain described above; a *Hin*dIII–*Hin*dIII fragment containing the immunoglobulin heavy chain locus 5′ enhancer, β-lactamase gene, and SV40 origin of replication and promoters (M. S. Neuberger and L. Riechmann, unpublished results); a *Hin*dIII–*Bam*HI fragment containing the coding sequence of a hygromycin-specific aminoglycoside phosphotransferase, SV40 t-antigen splice site and poly(A) sequences, excised from the plasmid pSV2*hyg (A. Smith, D. Strehlow and A. Miyajima, unpublished results). Next, the vector was partially digested with *Hin*dIII, and the *Hin*dIII site at the end of the hygromycin fragment removed by fill-in extension with DNA polymerase I (Klenow fragment) and religation. The human Cκ constant region was then introduced as a *Bam*HI fragment at the unique *Bam*HI site of the vector. (A genomic fragment containing the human J and Cκ gene segments (Hieter *et al.*, 1982) was first cloned as a 10 kb *Bam*HI fragment in pUC7 (Vieira & Messing, 1982), the J-segments excised as a 5 kb *Hin*dIII fragment and the vector religated. The 5 kb *Bam*HI fragment containing the Cκ coding sequence and the κ enhancer, was used in the construction.) The *Bam*HI site at the 3′ end of the Cκ constant region, and a *Hin*dIII site internal to the κ fragment were then eliminated by fill-in and religation of partial digests.

Construction of a reshaped anti-lysozyme antibody with human $\gamma 2$ constant region has been described (Verhoeyen *et al.*, 1988); our interest shifted to the $\gamma 1$ isotype, as it is more potent in activation of the complement cascade and of antibody-dependent cell-mediated cytotoxicity (Brüggeman *et al.*, 1987; Riechmann *et al.*, 1988). A clone of the human $\gamma 1$ constant region exons (Takahashi *et al.*, 1982), was provided as a 2 kb *Bgl*II fragment in the M13 phage tg131 (Kieny *et al.*, 1983) by M. Brüggemann. After *Hin*dIII digestion, fill-in and religation to destroy the internal *Hin*dIII site, the *Bgl*II fragment was introduced into the *Bam*HI-digested pSVgpt-HuV_HLYS-HulgG2 vector (Verhoeyen *et al.*, 1988) to replace the $\gamma 2$ constant region exons. Although the ligation destroys the *Bam*HI sites of the vector backbone, an internal *Bam*HI site (present in the polylinker of the M13-tg 131 vector) remains at the V-proximal end of the insert.

(d) *Construction of transfectoma lines*

Light and heavy chain constructs were introduced into myeloma cells by electroporation (Potter *et al.*, 1984), and stably transformed cells selected on the basis of drug resistance and cloned. Generally we co-transformed cells with both light and heavy chain constructs simultaneously, but we also tried transforming first with light chain, cloning the intermediate light chain producing transfectoma, then transforming with the heavy chain construct. The sequential method gave higher transfection frequencies, and was a convenient strategy in creating a family of antibodies with identical light chains, but similar yields of antibody were purified from clones obtained either through sequential or cotransformation. The level of antibody production by individual clones of cells varied widely: the better clones were identified by ELISA and picked for large scale growth.

Plasmids were purified from 1 l of bacterial culture by alkaline detergent lysis and CsCl/ethidium bromide equilibrium density gradient centrifugation (Ish-Horowicz & Burke, 1981). Heavy chain constructs were linearized with *Pvu*I, a step which increased transfection frequency by more than 10-fold. Light chain constructs do not have a convenient restriction site for linearization, so the circular form was used. The cell line NS0 (Galfré & Milstein, 1981), a myeloma which produces neither endogenous immunoglobulin chain due to abolition of heavy chain transcription and a defect in the κ transcript (Carroll *et al.*, 1988) was grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 110 mg sodium pyruvate/l, 100 mg streptomycin/l, and 100,000 units penicillin/l. Cells were harvested at a density of 10^6 cells/ml, and held on ice. 10^7 cells were suspended in 0·1 ml PBS or medium, placed in a sterile plastic 0·4 cm × 1 cm cuvette and mixed with 10 μg of each DNA construct. After several minutes, three 2 kV pulses from an Apelex (Bagneux, France) cell porator were applied, 1 s apart, then the cells were returned to ice. The electroporation mixture was washed into a flask with 25 ml medium, and grown overnight. An equal volume of selective medium was added, and the suspended cells distributed over a 24-well plate. For selection of hygromycin resistance alone, cells were exposed to a drug concentration of 0·4 g/l. For co-selection of hygromycin and mycophenolic acid resistance, culture medium contained 0·2 g hygromycin/l, 0·8 mg mycophenolic acid/l, and 0·25 g xanthine/l. As stock solutions of xanthine were made in 0·1 M-NaOH, an equivalent of HCl was also added to preserve the pH of the growth medium.

An antigen-based ELISA procedure was devised to facilitate screening of transformants. Microtitre plates (Dynatech) were coated overnight with 0·3 g lysozyme/l in 50 mM-$NaHCO_3$ (pH 9·6). The plates were washed with PBS, then blocked with 1% (w/v) bovine serum albumin in PBS for 5 min. 0·1 ml portions of culture supernatant were allowed to react for 2 h, and after washing, adsorbed antibody was quantified with peroxidase-conjugated rabbit anti-human IgG (Dakopatts, Denmark) diluted to

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2 μg/ml in 1% bovine serum albumin/PBS. After 1 h, plates were washed and substrates for the colour reaction added: 4 mM-H_2O_2, 1 mM-diammonium 2, 2′-azino bis (3-ethylbenzthiazoline sulphonic acid), in 0·1 M-citrate buffer (pH 4·5). For screening of light chain producing transfectomas, a similar procedure was used. Plates were coated with a 1:500 dilution of ascites fluid of the anti-human kappa hybridoma NH3/41 (Downie *et al.*, 1983), supplied by J. Jarvis. Peroxidase-conjugated rabbit anti-human kappa was used for quantitation.

Once visible colonies had appeared in the 24-well plates, after 2 weeks incubation, antibody production was assayed by ELISA. Cells from wells giving the strongest signals were cloned by limiting dilution in selective medium and regrowth on 96-well plates. The ELISA screening was repeated, usually on 20 or more clones, and the cell lines giving the strongest signals retained for permanent storage and large-scale growth for antibody production.

(e) *Antibody production*

Antibodies were purified to homogeneity from several litres of culture medium by affinity chromatography on both lysozyme-Sepharose and Protein A-Sepharose.

Sepharose CL-4B (Pharmacia), chilled in 1 M-Na_2CO_3, was activated for 2 min with 200 mg CNBr per ml resin, added as 1:1 solution in CH_3CN. The buffer was changed to 0·1 M-$NaHCO_3$, 0·5 M-NaCl (pH 8·5), and 5 mg lysozyme per ml. Sepharose was added, as a 1% solution in 10 mM-acetate, 40 mM-NaCl (pH 4·8). The slurry was stirred intermittently for 4 h and then residual imidocarbonate groups hydrolysed by overnight treatment with 0·1 M-$NaHCO_3$ (pH 10·9). To minimize the effect of leakage of the immobilized lysozyme during chromatography, the columns were washed with high-salt and elution buffers immediately before use. The lysozyme-Sepharose columns were used once and discarded. Protein A-Sepharose was washed with 0·1 M-citric acid before use; resin was in this case re-used.

Transfectoma cultures were grown from cloned stocks in flasks to 100 to 200 ml, in medium with both mycophenolic acid and hygromycin present, and 10% fetal calf serum. Each culture was then transferred to a roller bottle, and grown to 2 l with 5% serum with mycophenolic acid. Cells were grown until the culture medium was exhausted, leading to the demise of most cells. Cultures were cleared by centrifugation at 11,000 *g* for 15 min. Solid $(NH_4)_2SO_4$ was added slowly at a ratio of 0·313 kg/l of supernatant, and the mixture stirred for 1 h at 4 °C. Precipitate was harvested by a 45 min centrifugation dissolved in 20 ml phosphate buffered saline (125 mM-NaCl, 8·4 mM-NaH_2PO_4, 16·6 mM-Na_2HPO_4) (PBS) and dialysed against the same.

The dialysed protein was centrifuged to remove any insoluble material, and the reshaped antibodies applied to a 5 ml column of lysozyme-Sepharose, previously equilibrated in PBS. The column was washed extensively with high-salt buffer, 0·1 M-Tris, 0·5 M-NaCl (pH 8·5), and antibody eluted with 50 mM-diethylamine. 1 M-Tris·HCl (pH 7·4) was present in fraction collector tubes to neutralize the eluate. The eluate was then applied directly to a 5 to 10 ml Protein A-Sepharose column equilibrated in PBS. The columns were washed, eluted with 4 column vols of a gradient, 0·1 M-Na_3-citrate to 0·1 M-citric acid, and the antibody dialysed against PBS, 0·2 mM-EDTA.

For purification of the D1.3 antibody (mouse γ1 isotype) on Protein A-Sepharose, the sample was dialysed against either 3 M-NaCl, 0·1 M-glycine (pH 8·9), or against 0·1 M-Na_2HPO_4 (pH 8), before application to the column equilibrated in fresh dialysis buffer, and eluted with 0·1 M-citrate (pH 6).

Prior to storage, antibody was checked on an SDS/polyacrylamide gel (Laemmli, 1971) to verify purity, and an ultraviolet spectrum taken to ascertain concentration. Yields of purified antibody were approximately 15 mg/l of culture in the case of the hybridoma, and typically 2 to 3 mg/l for the transfectoma lines. Antibody solutions were filter-sterilized and stored at 4 °C under N_2 in acid-washed septum-top vials.

(f) *Physical methods*

The conditions chosen for all physical measurements were a temperature of 20 °C and a standard buffer of 25 mM-NaH_2PO_4, 125 mM-NaCl, 0·2 mM-EDTA (pH 7·0). Ultraviolet extinction coefficients ($\varepsilon_{280\,nm}$) were determined for the mouse and reshaped antibodies to facilitate routine, accurate determination of concentration. Quantitative amino acid analysis was performed on samples of known optical density, with norleucine added prior to hydrolysis as an internal standard. Correlation of the observed amino acid composition with that deduced from the encoded protein sequence gave $\varepsilon_{280\,nm}$ values of $2{\cdot}2 \times 10^5$ (M^{-1} cm^{-1}) for mouse and $2{\cdot}1 \times 10^5$ (γ2 isotype) or $1{\cdot}9 \times 10^5$ (γ1 isotype) for reshaped antibodies. Antibody preparations were quantified at the time of storage. Portions were withdrawn as needed and used directly, assuming that the concentration at the time of storage was unchanged. Hen egg lysozyme and bovine ubiquitin were from Sigma. These were dissolved in PBS and dialysed against the same buffer. Concentrations were determined spectrophotometrically, using $\varepsilon_{280\,nm}$ of 37,600 for lysozyme (Imoto *et al.*, 1972*a*) and 0·16 $(mg/ml)^{-1}$ cm^{-1} for ubiquitin (Ciechanover *et al.*, 1980).

(g) *Fluorescence spectroscopy*

Emission spectra revealed a large quench of native Trp fluorescence upon antibody-lysozyme complex formation; fluorescence measurements at fixed wavelengths were then used to determine the stoichiometry and approximate equilibrium constant.

A Perkin-Elmer LS-5B spectrofluorimeter interfaced to a Macintosh computer was used for all measurements. Of significance was the 50 Hz stroboscopic Xe light source, which gives a 150 W pulse, but has a root-mean-square intensity of 8 W, reducing photobleaching of samples to negligible levels. There was virtually no signal drift over the course of an experiment. Temperature control of the cuvette block was maintained by a thermostatted circulating water bath.

For emission spectra, an excitation wavelength of 280 nm was used, with a bandwidth of 10 nm; the emission bandwidth was 5 nm. Samples were scanned from 300 to 420 nm at 50 nm/min, with an 8 s time constant. In a typical set of measurements, a buffer sample was scanned to obtain a baseline. Antibody was added to 100 nM for the next scan, following which lysozyme was added in a negligible volume to 200 nM. A 3rd scan was taken after a 2 min wait for complex formation to occur. Similar measurements were made on a separate sample of buffer, then 200 nM-lysozyme alone. The spectra of protein samples were then corrected by numerical subtraction of the buffer blank.

In a typical titration, 15 samples of a concentrated lysozyme solution were added to a 3 ml sample of 10 to 200 nM-antibody, culminating in a molar ratio of 4 lysozyme/antibody (i.e. 2 lysozyme added per combining site).

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For higher antibody concentrations, a minimum interval of 2 min was maintained between adding lysozyme and reading the fluorescence. For lower concentrations, consideration was made of the length of time necessary for approach to equilibrium. An approximate relaxation time was estimated from any available kinetic and equilibrium data, and an interval several times that used, often in excess of 5 min. Titrations were most frequently done at an excitation wavelength of 280 nm and an emission wavelength of 400 nm. The excitation bandwidth was 10 nm. Emission bandwidths of 5, 10 or 20 nm were used, the choice for a given experiment depending on the magnitude of sample fluorescence. Each observation was obtained by averaging the fluorescence signal over a span of 16 s.

A source of systematic error in titrations was a progressive loss of fluorescence perhaps by adsorption to glass. This was especially compromising at high dilutions of antibody. For example, the fluorescence of a 10 nM solution of D1.3 antibody decreased 25% during a mock titration with samples of buffer. Use of ubiquitin as a carrier almost completely overcame this artifact. Ubiquitin has no tryptophan residues and a single tyrosine (Schlesinger *et al.*, 1975), hence is very weakly fluorescent. A concentration of 30 μg/ml gave a stable background, which was a fraction of the total fluorescence of the antibody and antigen. Discounting background, the fluorescence, F, of a mixture of antibody and lysozyme at equilibrium is described by the equation:

$$F = f_{Ab}rA + f_{Lys}L - f_{\Delta}\tfrac{1}{2}\{rA + L + K) - \sqrt{(rA + L + K)^2 - 4rAL}\}, \quad (1)$$

where A and L are the total (complexed and uncomplexed) antibody and lysozyme concentrations; r is the average number of combining sites per antibody molecule; f_{Ab} and f_{Lys} are the molar fluorescence of free antibody (on a per-site basis) and lysozyme; f_{Δ} is the molar fluorescence change occurring on complexation of lysozyme, a positive value indicating a net quench; K is the equilibrium constant for complex dissociation. A computer program for analysis of experimental data according to eqn (1) was written in Pascal and implemented on the Apple Macintosh computer. A and L were treated as independent variables, F as the dependent variable, and f_{Ab}, f_{Lys}, f_{Δ}, r and K were adjustable parameters optimized by the operation of the Marquardt least-squares algorithm on a first-order Taylor series expansion of eqn (1).

(h) *Rapid kinetics*

Measurement of the rate of antibody–antigen association was determined from a series of stopped-flow measurements under a pseudo-first-order regime. A commercial instrument of standard design (Hi-Tech Scientific, Salisbury, U.K.) was used for this work, in fluorescence mode. Light from a 75 W Xe lamp passed through a monochromator set at 280 nm, and thence *via* a light guide to the sample. Light emitted from the sample passed through a 320 or 360 nm cutoff (lower limit) filter before impinging on the photomultiplier. A Hewlett-Packard 310 computer fitted with an analogue-digital converter served for data capture and analysis, using a software package supplied by the stopped-flow manufacturer. Syringes and reactant reservoirs were bathed in water from a thermostatted circulating bath. The applied voltage on the photomultiplier was generally near 1·2 kV, giving a signal voltage of 1 to 6 V.

For measurement of on-rates, two 1 ml syringes were used, one with 100 nM-antibody, the other with 2, 4, 6 or 8 μM-lysozyme. A 20 nm excitation slit was used for high intensity. For a single observation, 50 μl portions of each reactant were mixed under maximum pneumatic pressure, and fluorescence data collected for a period chosen (after preliminary runs) to be approximately 6 half-lives. Ten or more transients were averaged prior to least-squares fitting to a simple exponential.

(i) *Equilibrium constant determination*

The method is based on a concept described by Segal (1975). A pseudo-equilibrium is established by a dilution in a stopped-flow instrument. The functional site concentration of an antibody preparation is first calibrated by titration with antigen. A mixture containing antibody at 11 times a notional pseudo-equilibrium concentration, on a site basis, and antigen at 22 times this concentration is allowed to equilibrate, then placed within a 0·25 ml stopped-flow syringe. A 2·5 ml syringe is filled with antibody alone, at 1·1 times the notional concentration. As the drive syringes are of proportional cross-sectional area, equal translation of their plungers configures an 11-fold dilution of the concentrated antibody and lysozyme mixture, and a 1·1-fold dilution of the free antibody solution, thus establishing a pseudo-equilibrium mixture with all 3 components, antibody, lysozyme and complex, approximating the notional concentration. For a single observation, a combined reactant volume of 100 μl was mixed, and data collected for 20 or 50 s.

A 10 nm excitation slit was employed to reduce photobleaching, and the longest available time constant, 100 ms, was used for signal stabilization. Traces for multiple identical runs were averaged, and fitted to an exponential. The amplitude of the exponential was taken as the fluorescence change, ΔF_{∞}, on relaxation of the initial mixture to a true equilibrium. The equilibrium constant, K, was determined by least-squares analysis as described in Results section (c).

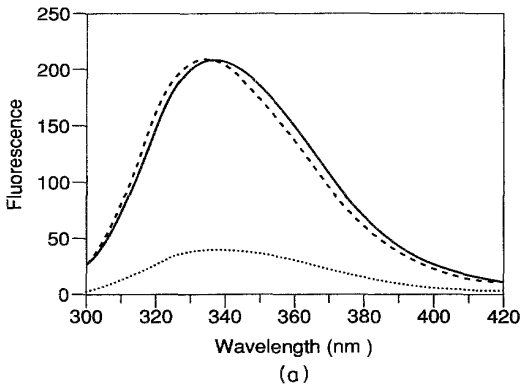
4. Results

(a) *Fluorescence spectroscopy*

Fluorescence emission spectra were taken of lysozyme, the set of anti-lysozyme antibodies, and antibody–antigen mixtures (see legend to Fig. 4). As is evident from the spectra in Figure 4(a), the fluorescence intensity observed from the mixtures was considerably less than the sum of intensities of the two separate components. Indeed, over much of the spectral range examined, the magnitude of this quench exceeded the fluorescence emission of free lysozyme, resulting in a decrease of fluorescence upon addition of antigen. The quench was not due to an "inner filter" effect as it was seen at low absorbance ($A_{280\,nm} = 0{\cdot}02$).

This quench at different wavelengths of emission is presented in Figure 4(b) in the form of a fluorescence difference spectrum, and also as a fraction of the total fluorescence. The peak of the difference spectrum, at 349 nm, is red-shifted from the maxima (<340 nm) of either antibody or antigen spectrum indicating that the quenched residues are in a polar environment (Van Duuren, 1961). Lysozyme has six Trp residues; however, more than 80% of the Trp fluorescence may be attributed to

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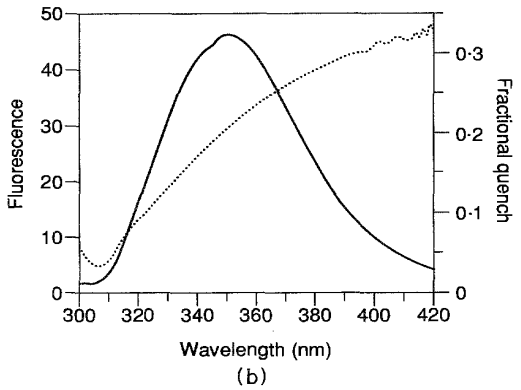


Figure 4. Fluorescence spectroscopy of D1.3 antibody and complex with lysozyme. (a) Fluorescence emission spectra of 100 nM D1.3 (continuous line), 200 nM-lysozyme (dotted line), and mixture at the same concentrations (broken line). (b) Difference and ratio spectra. A fluorescence difference spectrum (continuous line, left ordinate scale) was calculated by subtraction of the observed signal of lysozyme D1.3 mixture from the sum of the fluorescence of free D1.3 and lysozyme. The fractional quench (dotted line, right scale) was calculated as the ratio of this difference to the sum.

residues 62 and 108 (Imoto *et al.*, 1972*b*), neither of which is near the antibody–antigen interface. There are two light chain and four heavy chain Trp residues in cognate positions of the variable domains of D1.3 and the humanized antibodies. Of these, Trp92 of the light chain and Trp52 of the heavy chain make direct contacts to Arg125 and Asp119 of lysozyme, and are the likely origin of the quench effect. For the reshaped antibodies, the peak of the difference spectrum was also red-shifted, ranging from 350 to 363 nm.

The quench as a fraction of total fluorescence (Fig. 4(b)) increases monotonically with wavelength, reaching 30% at 400 nm in the case of D1.3. The reshaped antibodies yielded qualitatively similar spectra but the magnitude of the quench was generally lower (20%).

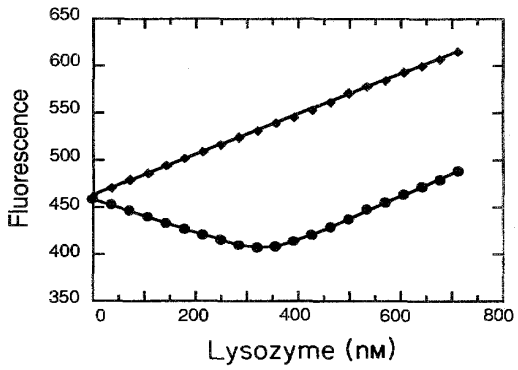


Figure 5. Titration of D1.3 antibody with lysozyme. Small samples of a concentrated lysozyme solution were added to 200 nM-D1.3 (—●—●—) and fluorescence emission measured at 390 nm. A control antibody (320 nM) with no specificity for lysozyme was titrated in parallel (—◆—◆—).

(b) *Fluorescence titrations and stoichiometries*

Antibody samples were titrated with lysozyme and fluorescence monitored at fixed wavelengths. Because a high fractional quench was more significant than the overall fluorescence signal in determining the sensitivity of these measurements, emission wavelengths of 390 to 400 nm were used, rather than the lower values corresponding to the maxima of the emission or difference emission spectra. Figure 5 shows the titration of D1.3 in parallel with a control antibody that does not bind lysozyme. Additions to the control gave a linear increase in fluorescence over the entire range of lysozyme concentrations. By contrast, as D1.3 was titrated, the fluorescence decreased until a stoichiometry of about two antigen molecules per antibody was reached, beyond which the signal rose linearly with the same slope as the control. Titrations of the reshaped antibodies gave similar data with varying curvature at the breakpoint. The observed stoichiometry of the antibodies with lysozyme, and absence of quench with the control, indicated that the spectral change arose from the combination of one molecule of lysozyme with each antigen binding site.

The curvature at the breakpoint depends on the affinity of the particular antibody for lysozyme. Fluorescence titration data were fitted to equation (1) to derive stoichiometries and equilibrium constants. The D1.3 sample in Figure 5 showed 1·82 antigen binding sites per antibody molecule, and other antibodies generally fell in the range 1·6 to 2·1 sites per molecule. The method gave highly reproducible stoichiometries. The equilibrium constants, however, were erratic, and often had large standard deviations. The pseudo-equilibrium relaxation method detailed in the next section thus was developed as a means of getting more reliable affinity data.

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(c) *Equilibrium constants*

Suppose that a 1 : 1 : 1 mixture of free antibody, free lysozyme, and antibody–lysozyme complex could be created artificially, and the concentrations of the components followed as the system relaxes to a true equilibrium. In the ideal mixture, if the pseudo-equilibrium concentration of the three reactants is greater than the dissociation constant, a net association of antibody and antigen would occur; if lower, there would be a net dissociation of the complex. When the pseudo-equilibrium concentration exactly matches the true equilibrium constant there would be no net change. By measuring changes in fluorescence on mixing, we could then estimate the equilibrium constant from the cross-over point, at which there is no change in fluorescence.

In practice we try to make the three equimolar reactants by mixing a concentrated lysozyme (2 molar equivalents): antibody (1 molar equivalent of binding site) solution with a dilute solution of free antibody (1 molar equivalent). Because the binding of antibody and antigen is not infinitely tight, we do not fulfil the ideal starting conditions: the actual concentration of complex, C, is given by the equation (2):

$$C = \tfrac{1}{2}\{(r\mathrm{A}+\mathrm{L}+K)-\sqrt{(r\mathrm{A}+\mathrm{L}+K)^2-4r\mathrm{AL}}\} \quad (2)$$

where the symbols are as for equation (1). The change in fluorescence ΔF_∞ occurring after mixing the two solutions is:

$$\Delta F = -f_\Delta\left\{\frac{C_0}{\mathrm{dil}}-C_\infty\right\} \quad (3)$$

where C_0 and C_∞ are given by equation (2), respectively, for the 2 : 1 cocktail prior to mixing and the full reaction mixture after complete re-equilibration, and d is the factor by which the concentrated solution is diluted. The fluorescence change is thus a function of K, and we can calculate K from a least-squares fit of experimentally determined ΔF values to equation (3).

Dissociation constants were determined for each of the reshaped antibodies by pseudo-equilibrium relaxation, and the values obtained are listed in Table 1. The results for HuLys0 are shown in Figure 6. In this case it was possible to approach the equilibrium from both directions (both positive and negative fluorescence changes), and the least-squares analysis indicates a dissociation constant of 260 nM. The precision of this technique in determining equilibrium constants proved superior to using fluorescence titration or the ratio of forward and reverse kinetic rate constants.

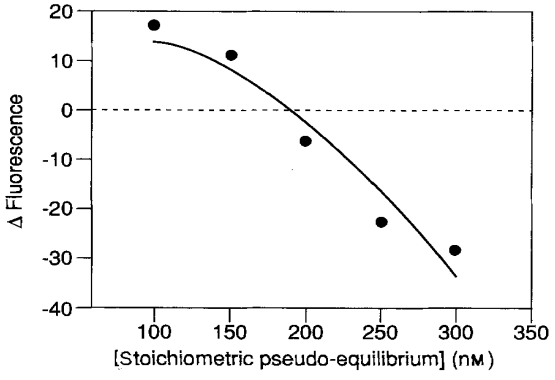


Figure 6. Determination of affinity constant of HuLys0 reshaped antibody for lysozyme by pseudo-equilibrium relaxation. Antibody and antibody with lysozyme solutions were mixed as described in the text, to give the "pseudo-equilibrium" concentration. This is a notional value, equal to the concentration of free antibody sites, free antigen and complexed antigen which would occur upon mixing in the limit of infinitely tight antibody–antigen interaction. The fluorescence change occurring after mixing is plotted in the ordinate. The curve is calculated from parameters derived by least-squares analysis of the experimental points.

HuLys0, the prototype reshaped antibody, has an affinity in the range expected for an antibody in a primary immune response, but nearly two orders of magnitude weaker than D1.3. Framework substitutions effected significant increases in antigen affinity. The data in Table 1 are presented in a

Table 1
Physical constants of anti-lysozymes

Antibody	k_{on} (mol^{-1} L s^{-1}/10^6)	K_d (nM)	Residue substitutions				Isotype
			V_K : 71	V_H : 27	29	71	
D1.3	1·39 (0·02)	3·7 (1·8)	Y	F	L	K	—
HuLys0	0·69 (0·02)	260 (16)	F	S	F	V	γ2
HuLys3	1·18 (0·03)	64 (13)	Y	S	F	V	γ2
HuLys6	1·45 (0·12)	48 (9)	Y	F	F	V	γ1
HuLys7	1·33 (0·15)	77 (4)	Y	S	F	V	γ1
HuLys8	1·26 (0·10)	26 (5)	Y	F	L	V	γ1
HuLys9	0·63 (0·03)	120 (7)	F	F	L	V	γ1
HuLys10	1·33 (0·12)	18 (9)	Y	F	F	K	γ1
HuLys11	1·31 (0·12)	14 (2)	Y	F	L	K	γ1

Parenthetical values are standard errors.

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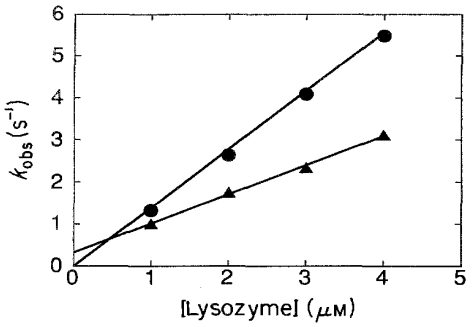


Figure 7. Kinetics of antigen binding. 50 nM-D1.3 (—●—●—) or HuLys0 (—▲—▲—) was reacted with excess lysozyme in a stopped-flow instrument. The observed pseudo-first order rate constant is plotted against the corresponding lysozyme concentration. The slope of the regression lines corresponds to the second order rate constant for antibody–antigen association.

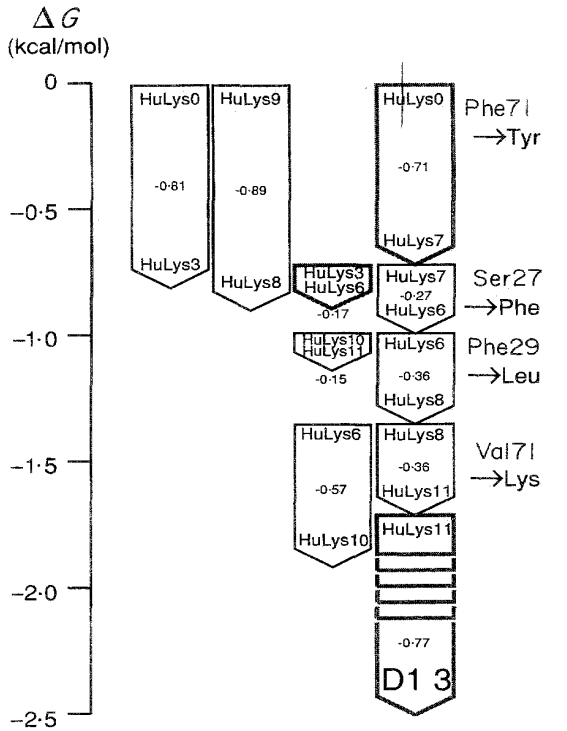


Figure 8. Energetic effect of framework substitutions. Differences in free energy of association were calculated for pairs of antibodies (using $\Delta G = -RT \ln K_1/K_2$) from the equilibrium constants in Table 1. Each of the boxes denotes the difference in free energy of lysozyme binding resulting from mutation. The boxes can be built into a train reflecting the energetic changes of a sequential transformation of the weakest affinity antibody, HuLys0, into the strongest, D1.3. A shaded outline denotes molecules differing in constant region isotype. Several of the comparisons are not strictly single-site mutations. The heavy chain mutation Phe29 to Leu was co-ordinated with 2 others, such that reshaped antibodies had either the sequence Thr28-Phe29-Ser30 or Ser28-Leu29-Thr30. The side-chains of residues 28 and 30 lie on the surface and they do not appear to interact with the remainder of the antibody (Fischmann *et al.*, 1991).

different form in Figure 8. A free energy scale denotes changes in free energy of association with antigen, starting from HuLys0. The progression HuLys0-7-6-8-11 represents a systematic addition of single-step changes in the variable domains, ignoring changes in isotype from human $\gamma 1$ to $\gamma 2$ antibodies (HuLys0 to HuLys7) and changes at V_H 28 and 30 (HuLys6 to HuLys8) which are probably irrelevant to binding affinity (see Discussion). A final step in Figure 8, from HuLys11 to D1.3, denotes the energetic gain that would accrue from a further 42 mutations to restore the mouse framework regions. The four mutations introduced into the human framework regions thus recoup two-thirds of the antigen-binding free energy lost in the initial CDR transplant.

(d) *Kinetics of antigen binding*

It was possible to follow the kinetics of binding from the fluorescence changes on complex formation. Antibody was mixed with a 10 to 40-fold excess of lysozyme per site, and the reaction followed for periods of up to two seconds. The resulting fluorescence change in all cases followed a simple exponential allowing a pseudo-first order rate constant to be abstracted by least-squares analysis. The observed rate constants were in turn plotted against the concentration of lysozyme used (Fig. 7), and the slope of the resulting line taken as the second order rate constant for bimolecular association (on-rate). On-rates are listed along with equilibrium dissociation constants in Table 1. The D1.3 antibody binds lysozyme with a rate constant of $1{\cdot}4 \times 10^6$ l mol^{-1} s^{-1}, as do several of the reshaped antibodies. This indicates that the initial recognition event is identical, despite differences in affinity that are largely controlled by the off-rate. However, two of the reshaped antibodies have slower on-rates, correlating with light chains with Phe71.

In Figure 7 the ordinate intercept of the line corresponds to the off-rate. For D1.3, the line appears to intersect the origin (due to slow off-rate) and the off-rate cannot be determined in this way. However, for HuLys0, the off-rate is faster, and the intercept is displaced further from the origin and can be determined. The off-rate values that could be determined in this way (HuLys0 and HuLys9) were consistent with those calculated from k_{on} and K (determined independently as above). The D1.3–lysozyme complex dissociates with a calculated half-life of 134 seconds, contrasting with a four second bound lifetime for HuLys0, the weakest binder.

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5. Discussion

Here we have described the expression of anti-lysozyme reshaped antibodies (Jones *et al.*, 1986; Verhoeyen *et al.*, 1988; Riechmann *et al.*, 1988) as human $\gamma 1$, κ isotypes in myeloma cells. Variable region expression cassettes, including Ig promoter, signal and splice sites, were built in M13 vectors to facilitate oligonucleotide-directed mutagenesis. These cassettes were readily cloned into pSV vectors (Mulligan & Berg, 1980; Neuberger, 1983) carrying human $\gamma 1$ and κ genes for expression of antibody. These M13 and pSV vectors have general utility, and aspects of this work have already been reported by Riechmann *et al.* (1988) and Orlandi *et al.* (1989) as unpublished results.

We also describe several methods, using fluorescence quench on antigen binding, for the determination of antibody affinity, kinetics and stoichiometry of binding. Hitherto the use of fluorescence quench to study the interaction of protein antigens with antibody has involved the chemical coupling of spectral probes to antibody or antigen. Here we use the changes in fluorescence of tryptophan residues on forming the antigen–antibody complex to follow the interaction. This allowed the accurate determination of equilibrium constants of the antibody–lysozyme interaction, and this proved essential for following the small energetic changes between mutants.

Antibody variable domains comprise a framework of β-sheet surmounted with antigen-binding loops that can be transplanted between antibodies. However, some amino acids of the framework appear to make important interactions with the loops (Chothia & Lesk, 1987). We attempted to dissect their contribution by "rebuilding" the antigen-binding affinity of a reshaped anti-lysozyme antibody ($K = 260$ nM), and have focussed on four framework substitutions, Vκ Phe71 → Tyr, V_H Ser27 → Phe, Phe29 → Leu and Val71 → Lys. The reasons for designing these substitutions are given in Materials and Methods. We found that the lost affinity could be rebuilt to 14 nM by introducing all these mutations, to within a factor of four of the original mouse antibody (3·7 nM). However, each mutation alone made a small contribution to the enhanced affinity. Vκ Phe71 → Tyr had the greatest effect and was unique in increasing the rate constant for association; the other changes only affected the rate of dissociation of the antibody–antigen complex.

The residues 27, 29 and 71 in the heavy chain form a contiguous triad, in which Phe27 and Leu29 side-chains and the hydrophobic portion of the Lys71 side-chain interact together, and with the loops CDR 1 and 2 (Amit *et al.*, 1986). The energy diagram (Fig. 8) suggests that these residues do not behave independently on supporting the loops in antigen binding. For example, comparing the two sites at residues 29 and 71 of the heavy chain, the energetics of the changes (Phe29 → Leu) or (Val71 → Lys) depend on the precise residue at the other site. However, comparing the two sites at residue 27 of the heavy chain and 71 of the light chain, the energetics of the change (Phe71 → Tyr) are independent of the precise residue at the other site.

Table 2
Residues in the "Vernier" zone (Kabat numbering)

Heavy chain	Light chain
2	2
27–30	4
47–49	35–36
67	46–49
69	64
71	66
73	68–69
78	71
93–94	98
103	

These residues are examples of a more general subset of framework residues acting as a "Vernier" zone (Fig. 1(c)), which may adjust CDR structure and fine-tune the fit to antigen. In Table 2 we list framework residues forming a layer underlying the CDRs and that are positioned to exert these effects. From inspection of known crystal structures, such roles have been ascribed to heavy chain residue 71 (Tramontano *et al.*, 1990), and implicitly to residues 27 to 30, which form part of an antigen-binding loop (Chothia & Lesk, 1987). Such roles are also consistent with the altered binding properties of antibody mutants. Independent studies of anti-digoxin (Panka *et al.*, 1988; Novotny *et al.*, 1990) and anti-phosphorylcholine (Chien *et al.*, 1989) antibodies drew attention to the specific interaction of heavy chain framework residue 94 with heavy chain CDR residue 101.

The Vernier zone has an obvious consequence for the design of humanized antibodies. Although grafting CDRs onto a single set of acceptor frameworks may well transfer the antigenic specificity of the donor antibody (Jones *et al.*, 1986), ideally the Vernier residues should also be matched. Indeed substitutions of these residues have been shown to be important to restoring the affinity in CDR grafted antibodies, for example in heavy chains residue 27 in the antibodies CAMPATH-1 (Riechmann *et al.*, 1988) and D1.3, residue 71 in D1.3, residue 94 in anti-RSV (Tempest *et al.*, 1991); in light chains residue 71 in D1.3.

In view of the structural role for residues of the Vernier zone in supporting CDR loop conformations and/or their relative dispositions, alterations at these points may have some role in affinity maturation. Arg94 of the V-186.2 heavy chain of the anti-nitrophenacetyl response is frequently changed to Thr (Blier & Bothwell, 1987; Allen *et al.*, 1988). Likewise residue 36 of the Vκ-Ox1 anti-phenyloxazolone light chain consistently mutates from Tyr to Phe (Griffiths *et al.*, 1984). This residue appears to support the local CDR structure and to interact

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directly with hapten (Alzari *et al.*, 1990). In the T15 anti-phenyl-oxazolone heavy chain, residue 49 commonly changes from Gly to Ala or Val (Berek *et al.*, 1985). Furthermore, we note that closely related germline genes (see Pascual & Capra, 1991, for a compilation of sequences) can differ at one or several of these sites, suggesting that variability of these residues may also be a component of the diversity of structures found in the primary antibody repertoire.

We thank Roberto Poljak for D1.3 co-ordinates, Martine Verhoeyen for the D1.3 nucleotide sequence, and Marianne Brüggeman, Philip Leder, Michael Neuberger and Andrew Smith for vector parts. We also acknowledge Cyrus Chothia, Tom Creighton and César Milstein for valuable discussions. J.F. was a Fellow of the Jane Coffin Childs Fund for Medical Research, and a Merck Fellow.

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PROTEIN DRUG STABILITY: A FORMULATION CHALLENGE

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Abstract | The increasing use of recombinantly expressed therapeutic proteins in the pharmaceutical industry has highlighted issues such as their stability during long-term storage and means of efficacious delivery that avoid adverse immunogenic side effects. Controlled chemical modifications, such as substitutions, acylation and PEGylation, have fulfilled some but not all of their promises, while hydrogels and lipid-based formulations could well be developed into generic delivery systems. Strategies to curb the aggregation and misfolding of proteins during storage are likely to benefit from the recent surge of interest in protein fibrillation. This might in turn lead to generally accepted guidelines and tests to avoid unforeseen adverse effects in drug delivery.

During the past two decades, recombinant DNA technology has led to a significant increase in the number of approved biotechnology medicines and a shift away from the production of biologically active materials (biologics) on the basis of animal or human material, and towards cloning and fermentation. A recent survey lists 324 biotechnology medicines, either in human clinical trials or under review by regulatory agencies. These drugs cover nearly 150 diseases, including cancer, infectious diseases, autoimmune diseases and AIDS/HIV[1]. A substantial number of these substances are proteins. This development will expand the list of medicines and open the way for more and better treatments to the benefit of patients.

This general trend accords with the vision of several analysts, including IBM Business Consulting Services[2]. According to this view, many of the new medicines will be based on the recombinant DNA expression of proteins rather than organic chemistry, because biologics in general are expected to be less toxic and to behave more predictably *in vivo*. Biotechnological medicines could therefore potentially reach the market faster than chemical entities developed through traditional methods. On the other hand, the decoding of the human genomic and proteomic maps is anticipated to lead to discoveries in molecular medicine that could revitalize the development of small-molecular synthetic drugs[3,4]. In many respects, the efficacy and safety requirements of biologics are similar to the requirements for small, chemically synthesized drugs. However, owing to the biological origin and macromolecular structure of biologics, there is particular focus on contamination of biologics with other biological impurities, such as viruses, as well as conformational changes introduced either during production of the bulk substance or the final formulation. Well-documented and validated biological, physical and chemical methods are important tools for securing the quality and safety of biologics.

Several challenges confront pharmaceutical scientists involved in the development of biotechnological medicines, such as proteins. The successful formulation of proteins depends on a thorough understanding of their physico-chemical and biological characteristics, including chemical and physical stability, immunogenicity and pharmacokinetic properties. The therapeutic activity of proteins is highly dependent on their conformational structure. However, the protein structure is flexible and sensitive to external conditions, which means that production, formulation and handling of proteins needs special attention in optimizing efficacy and safety, including minimized immune responses. The primary focus of this review is on the challenges related to quality and safety issues that arise in the development of protein-based medicines.

The chemical and physical stability of proteins can be compromised by external factors such as pH, temperature

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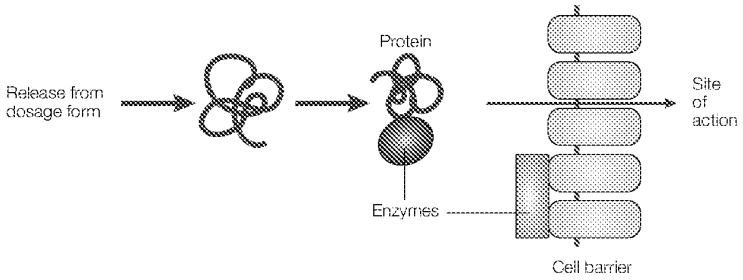


Figure 1 | **Barriers to biomacromolecular drug transport.** The major obstacles for efficient transport to the site of action are enzymatic degradation and biological membranes.

and surface interaction, as well as by contaminants and impurities for excipients, and so on[5,6]. Several recent reviews discuss the importance of protein stability in formulation development and the immunogenicity of proteins from a pharmaceutical perspective[5,7]. In addition, several recent reviews focus on conditions leading to undesirable changes in the chemical structure of proteins[8,9].

Delivery challenges

Oral administration of medicines is the preferred and most widely used route of administration. However, this route is generally not feasible for the delivery of macromolecules such as proteins. The inherent instability of proteins in the gastro-intestinal tract, as well as the low permeability across biological membranes due to the high molecular mass and polar surface characteristics of proteins, implies that proteins for systemic treatment should be administered PARENTERALLY; however, efforts are being made to improve bioavailability through alternative routes of administration, for instance, by the nasal or pulmonary route[10,11]. Nevertheless, bioavailability through the various non-parenteral routes of administration is low and normally insufficient for an effective systemic effect. The obstacles for efficient delivery to the site of action can broadly be categorized as either various enzymatic barriers that the protein encounters in moving from the administration site to the site of action, or physical barriers to effective transport, such as epi- and endothelial cell linings (FIG. 1).

The pharmaceutical and PHARMACOKINETIC properties of proteins can be optimized by different approaches — for example, by mutagenesis, chemical modification or by designing specific drug-delivery systems. However, most protein-based medicines today are still formulated as suspensions or aqueous solutions either in a ready-to-use form or as a lyophilized product for reconstitution.

Substitution and chemical modification

Proteins for therapeutic use can be chemically modified in several ways — for example, by mutating one or more amino acids (that is, creating a protein analogue) or by acylation or PEGylation. These modifications can be used to optimize the pharmacokinetic properties of the protein, but care must always be taken not to reduce their biological efficacy.

Protein analogues. The development of rapid-acting monomeric insulin analogues provides an excellent example of the analogue approach[12]. The basic idea behind this development was that the transport rate from the subcutaneous injection site of administration across the biological membrane to the systemic circulation would be increased if the self-association characteristics of insulin were shifted from hexameric insulin to di- or monomeric insulin (FIG. 2). By mutating amino acids involved in self-association, this approach was proven to be feasible. Monomeric insulin analogues with a more rapid time-action profile than human insulin are now on the market (for example, insulin lispro (Eli Lilly) and insulin aspart (NovoRapid; Novo Nordisk)).

Recombinant human interleukin-2 (IL-2) is another example in which the therapeutic effect is improved through molecular design. The commercially available non-glycosylated IL-2 analogue aldesleukin (Proleukin; Chiron) is a des-alanyl-1 analogue of human IL-2 in which cysteine in position 125 has been replaced by serine. The experimental IL-2 analogue BAY 50-4798 (Bayer) is another example of a genetically engineered protein with potentially improved therapeutic effect.

Acylation. Chemical attachment of fatty acids to exposed residues on the protein surface can in some cases increase the affinity of the protein to serum albumin sufficiently to increase its circulation time in the blood[13,14]. The effect of acylation is naturally greater for smaller proteins and peptides because of the greater relative increase in hydrophobicity. For example, the acylated insulin analogue [Lys.sup.B29]-tetradecanoyl des (B30) human insulin, insulin detemir (Novo Nordisk A/S) is long-acting and has recently been approved by the regulatory agencies in some countries. The principle of acylation has also been applied successfully to other proteins — for example, glucagon-like peptide 1 (GLP1) (REF. 15) and interferon-α[16], as well as for peptides such as desmopressin[17]. Whereas the acylation of insulin and GLP1 is site-specific, the acylation of interferon is less specific. This potential heterogeneity of acylated interferon could eventually prove to be a problem both from an efficacy and a safety point of view.

Language to consider since we may argue that claims to a "solution" are not lyophilized compositions.

PARENTERAL
A substance that is introduced into the body anyway except by mouth.

PHARMACOKINETIC
The study of the absorption, distribution, metabolism, excretion and interactions of a drug.

PEGylation
The covalent binding of polyethylene glycol (PEG) to a protein.

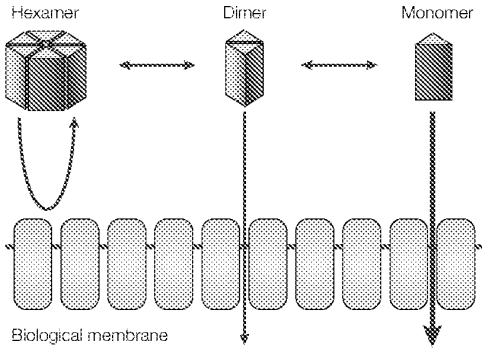


Figure 2 | **Transport of insulin across biological membranes.** Insulin monomer has the highest flux.

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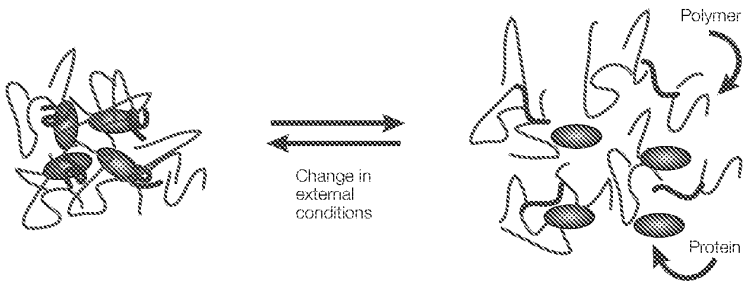


Figure 3 | **Schematic illustration of protein release from a polymer sensitive to external stimuli, such as change in pH, ionic strength and temperature.** Swelling of the polymer releases the entrapped protein.

PEGylation. In general, PEGylation reduces the plasma clearance rate by reducing the metabolic degradation and receptor-mediated uptake of the protein from the systemic circulation. PEGylation also improves the safety profile of the protein by shielding antigenic and immunogenic EPITOPES[18]. However, it is important to realize, both from an efficacy and safety point of view, that PEG polymers consist of a mixture of polymers with different molecular mass; in addition, larger proteins will have several sites that are accessible to PEGylation. Owing to this potential product heterogeneity, the clinical and pharmaceutical documentation required for drug approval could be even more demanding than for non-modified proteins. PEG-interferon-α is a commercial example in which the pharmacokinetic profile is improved by PEGylation, which enables less frequent administration and results in improved efficacy with a similar side-effect profile[19]. On the other hand, mono-PEGylated epidermal growth factor (EGF) with PEG 3400 at Lys28 and Lys48 was significantly less active than an EGF isomer PEGylated at the amino terminus in an *in vitro* assay for mitogenic activity[20]. Several excellent reviews are available on these specific types of derivative. A recent issue of *Advanced Drug Delivery Reviews* is devoted to PEGylation[21].

Drug-delivery systems. Entrapment and encapsulation are the most widely used formulation principles adopted for protein-delivery systems. Polymeric drug-delivery systems, such as hydrogels, nanocapsules and microspheres, and lipid-based drug-delivery systems such as liposomes and solid lipid nanoparticles, are all examples of protein-delivery systems (FIG. 3).

Hydrogels are crosslinked hydrophilic polymers that form three-dimensional networks, which can either be synthesized to degrade at a certain rate or to respond to several physiological stimuli present in the body, such as pH, ionic strength and temperature. Both principles are used for the controlled release of proteins[22–25]. These hydrogels can be formulated either as microspheres or as *in situ* forming delivery systems. The *in situ* forming systems could have several advantages over conventional microspheres, such as ease of administration and less complicated and potentially harmful manufacturing conditions appropriate for sensitive drug molecules such as proteins[26].

Examples of lipid-based formulations with the potential for serving as delivery systems for macromolecules such as proteins include liposomes, solid lipid nanospheres and water-in-oil emulsions[27–29]. The incorporation and release of proteins from liposomes, for instance, can be controlled by the physico-chemical characteristics of the protein — that is, the hydrophobic surface area, which can be modified, for example, by acylation[30] or by constructing lipid bilayers that are sensitive to triggered degradation by enzymes such as secretory phospholipase A2 that are present in inflammatory tissues and certain cancerous fluids[31]. Representative examples of modifications and delivery systems are given in TABLE 1.

Immunogenicity of therapeutic proteins. The ability to produce highly purified proteins that are identical or nearly identical to endogenous human proteins is often considered as the key to avoiding T- and B-lymphocyte reactivity during treatment, because patients are expected to be immune tolerant to their own proteins. However, although the level and incidence of immune responses is low, most of these proteins have been shown to be immunogenic, and in some cases have even led to serious safety problems and inhibition of the therapeutic effect due to neutralizing antibodies. An example is antibody formation after long-term treatment with recombinant interferons in patients with multiple sclerosis, which indicates that the presence of neutralizing antibodies against interferons reduces the clinical effect of the drug[32,33]. Recombinant human erythropoietin is another example in which the neutralizing antibodies react against erythropoietin and cause pure red-cell aplasia[34]. It has been suggested that the reason for anti-erythropoietin antibody production seen in patients might be related to the production process of the hormone[35]. A formulation change introduced in the late-1990s has also been proposed as a plausible reason for the induction of antibodies that neutralize the endogenous erythropoietin. A recent study indicates that this formulation contains micelle-associated erythropoietin, which could be a risk factor for the development of antibodies[36]. The difficulties in detecting structural changes in proteins in more complex pharmaceutical formulations is a major challenge for formulation scientists responsible for the development and quality of biopharmaceutical medicines[37].

In general, it is the protein structure that can trigger an immune response during treatment. Proteins are complex and flexible molecules, and even a small change at a particular site can result in a major change in the overall structure (TABLE 2). For instance, recombinant mutated allergens with a modified surface topography, but that retain an α-carbon-backbone folding pattern, have been shown to retain the capacity to induce allergen-specific immune responses, but with different anaphylactic potential[38]. This shows that although the surface structures positioned outside the mutated regions are retained, the introduction of mutations that change both the topography and the charge

EPITOPE
An alternative term for an antigenic determinant. These are particular chemical groups on a molecule that elicit a specific immune response.

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Table 1 | **Modified proteins and protein-delivery systems approved for marketing**

Product (company)	Drug	Modification/delivery system	Administration route
Proleukin (Chiron)	Aldesleukin	Analogue	Intravenous
Humalog (Eli Lilly)	Insulin lispro	Analogue	Subcutaneous
NovoRapid (Novo Nordisk)	Insulin aspart	Analogue	Subcutaneous
Neulasta (Amgen)	PEGinterferon α-2a	Mono-pegylated	Subcutaneous
Pegasys (Roche)	Pegfilgrastim	Mono-pegylated	Subcutaneous
Somavert (Pharmacia)	Pegvisomat	Multi (4–6)-pegylated	Subcutaneous
Levemir (Novo Nordisk)	Insulin detemir	Mono-acylated	Subcutaneous
Nutropin Depot (Genentech)	Human growth hormone	PLGA microspheres	Subcutaneous
InductOs (Wyeth (MDT))	Bone morphogenic protein 2	Absorbable collagen sponge	Implanatable medical device

PLGA, poly(lactide-co-glycolic acid)

distribution can be sufficient to affect the immunogenicity of the protein (FIG. 4). However, it is normally difficult to relate a particular change in protein structure to a change in immunogenicity.

Two is a crowd: coping with aggregation

During processing and formulation of the drug product, the protein is exposed to conditions that could have significant effects on its chemical and physical stability, and lead to aggregation and ultimately precipitation[9,39–41]. It is therefore important to understand the circumstances by which protein stability is compromised. The main factors are shear/shaking, temperature, pH and protein concentration (see REF. 39 for a detailed overview). Shear forces encountered by vortexing can partition proteins to the air–water interface, which encourages partial unfolding on exposure to the more hydrophobic air phase[41,42]. This will be even more pronounced if the second phase involves an organic solvent such as chloroform[43]. Elevated temperatures[44], changes in pH or intermediate denaturant conditions[45] can also favour the formation of such states. Small, single-domain proteins usually require extreme conditions to unfold, but for large, multidomain proteins, a few 'weak links' unravelling under relatively gentle conditions can be sufficient to initiate aggregation. Partially unfolded states are much more susceptible to aggregation than the native or unfolded state, due to the exposure of contiguous hydrophobic regions that are buried in the native state or absent in the denatured state[40,46].

Although aggregation is fundamentally bi-molecular, the dependence on concentration will reflect the mechanism of the process. If unfolding of the native state is rate limiting, aggregation kinetics can be pseudo-first order[47,48], whereas there is an inverse dependence for processes involving air–water interfaces[49]. Initial dimer formation is seen with colony stimulating factor[50], whereas lysozyme aggregation follows higher-order kinetics[51]. Aggregation often shows high activation barriers, which make the process slow, irreversible and kinetically controlled. This means that the handling history of a sample can have a disproportionate role in the outcome. Irreversible aggregation, caused by disulphide-shuffling or stable hydrophobic association, can have a direct impact on drug potency, immunogenicity[47] and the unfolded protein response[52,53]. Reversible aggregation, however, can also be problematic during drug administration, if dissociation is slow on the physiological time scale. Slow dissociation kinetics can be a consequence of crowding effects in the body, which favour the aggregated state even after dilution[54,55].

The protein chain is chemically complex and can assume an astronomical number of possible conformations, the relative stabilities of which are very sensitive to pH, ionic strength and temperature. This creates a large number of 'conformational end stations' — including both those that are monomeric and oligomeric — that depend on environmental conditions (FIG. 5). The aggregates are not just featureless heaps; it has been known for a long time that aggregation is generally quite specific[56], involving well-defined oligomerization interfaces. Probably the most important — and certainly most well-characterized — aggregation state is the amyloid fibril, associated with neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease and many others. Curiously, fibrillation is exploited in a wide range of organisms, ranging from biofilm in *Escherichia coli*[57] to melanosome formation in humans[58]. It has been suggested that all proteins can form fibrils with the same structural characteristics, namely a cross-β structure and parallel β-helices[59], irrespective of the native structure and primary sequence[60]. This presents a potential hazard during production and/or storage. Fibrillation is particularly encouraged under moderately destabilizing conditions, such as pH below 3 or above 10, temperatures above 40° C and/or intermediate denaturant concentrations, which allow the protein better access to other conformations while retaining some structure.

Table 2 | **Potentially immunogenic protein modifications**

Modification	Effect
Engineered modifications	
Amino-acid sequence	Human versus analogues and non-human proteins
Chemical modification	Acylation, PEGylation
Pharmaceutical formulation	Lyophilization, micro-encapsulation
Unwanted modifications during processing, production and storage	
Chemical degradation	Deamidation, oxidation
Physical degradation	Denaturation, aggregation, fibrillation, misfolding

PEG, polyethylene glycol.

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Fibrillation is generally modelled as a nucleation-dependent process, in which the nuclei accumulate during the lag phase without any bulk conformational changes, followed by the rapid accumulation of fibrillated protein as the nuclei are extended[61]. The lag phase is typically very long, mainly because the higher-order equilibria leading to nuclei are unfavourable at low protein concentrations. This makes the process very sensitive to protein concentration; for sickle-cell haemoglobin fibrillation, concentration dependencies of up to 50 have been predicted and observed[62]. Simulating aggregation *in silico*[63,64] or setting up mathematical models[65] can be useful to predict and prevent problems in protein production, but it remains challenging to incorporate all the environmental factors that impinge on the process. Aggregation is fundamentally a deviation from the 'productive' folding pathway, and it has been proposed that the individual energy landscapes or funnels describing protein folding should really be represented as 'double funnels', the second of which represents intermolecular interactions of partially folded states (FIG. 6)[66].

Intermolecular β-strand formation can be a powerful driving force for aggregation, and the propensity to form β-strands generally correlates positively with aggregation, in contrast to that of α-helices[67]. However, it has recently been suggested that α-helices can contribute to aggregation through the formation of coiled-coil contacts[68], although this is unlikely to be a general mechanism. It is now possible to predict absolute aggregation rates and the effect of different mutations on aggregation using simple physico-chemical approaches[69–71], such as changes in secondary structure propensities, hydrophobicity, patterns of alternating hydrophobic–hydrophilic residues, pH, ionic strength, concentration, and so on. For example, the sequential location of hydrophobic amino acids controls fibrillation of the amyloid β (Aβ) peptide[72] and insulin[73]. Aggregation propensities can even be predicted at the level of individual residues to pinpoint aggregation hot spots[69]. Key 'gatekeeper' residues are sometimes involved in stabilizing/destabilizing such interactions, so a few mutations can easily tip the balance towards the monomeric form[74,75]. However, it is characteristic of the complexity of the reactions leading to aggregation that a simple parameter such as stirring has yet to be incorporated into these models.

The lag time is probably the most important parameter from a formulation point of view. Unfortunately, it is very difficult to predict, not only because of the stochastic nature of the nucleation processes that can give rise to a lag phase, but also because of the complexity of the mechanism involved. It is common to assume that lag times represent a nucleation process[61], but this is not always the case[76]. Lag phases can also be observed even with a supply of preformed fibril seeds[76] (J.S. Pedersen and D.E.O., unpublished observations). In such cases, fibrillation is more likely to be promoted by small fibrils fragmenting to smaller entities that can capture more monomers before they fragment again, leading to the exponential growth of monomer-ensnaring fibrils[77,78]. Here, the lag phase does not have a simple physical interpretation, but simply reflects that the amount of amyloid is too low for detection in the lag phase. For polyglutamine sequences involved in Huntington's disease, the nucleus is actually a monomer[79], revealing that the rate-limiting step is not the assembly of an oligomeric species but a conformational change occurring at the monomer level.

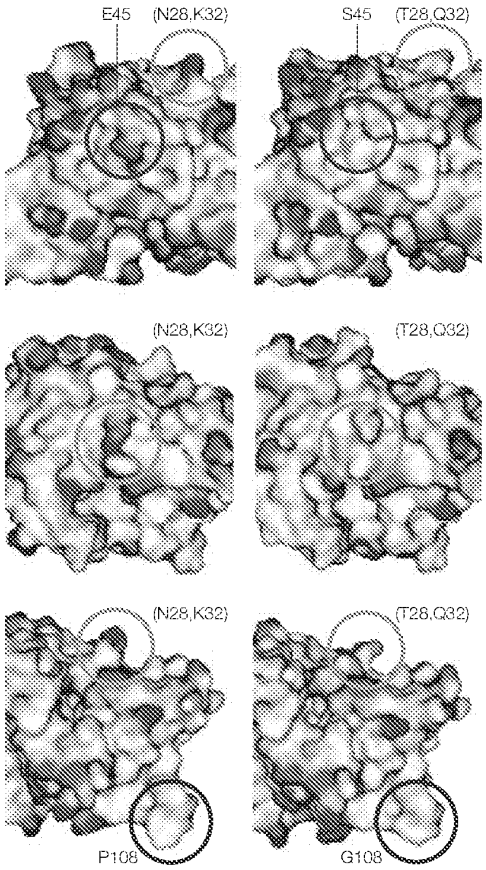


Figure 4 | **Protein structure and immunogenicity.** Comparison of molecular surfaces coloured according to electrostatic potential. Introduction of amino-acid mutations change both the topography and the polarity locally, whereas surface structures outside the mutation sites are retained. Adapted, with permission, from REF. 38 © (2004) American Association of Immunologists.

In cases in which seeding has been shown to accelerate the process, such as for the Aβ peptide[80], it might provide some relief to the biotechnological community to know that spurious fibrillar contaminants of another protein need not wreak havoc. The seeding process is very specific and demands very close sequence[81] and enantiomer[82] correspondence between the soluble monomer and the fibril; even point mutations can completely abolish seeding ability[83]. Nevertheless, it should also be noted that fibrillating proteins can form fibrils that differ significantly at the molecular level, simply by varying growth conditions such as stirring/aggregation[84], temperature or ionic strength (J.S. Pedersen and D.E.O., unpublished observations), and these differences can be propagated from one generation to the next. This polymorphism is

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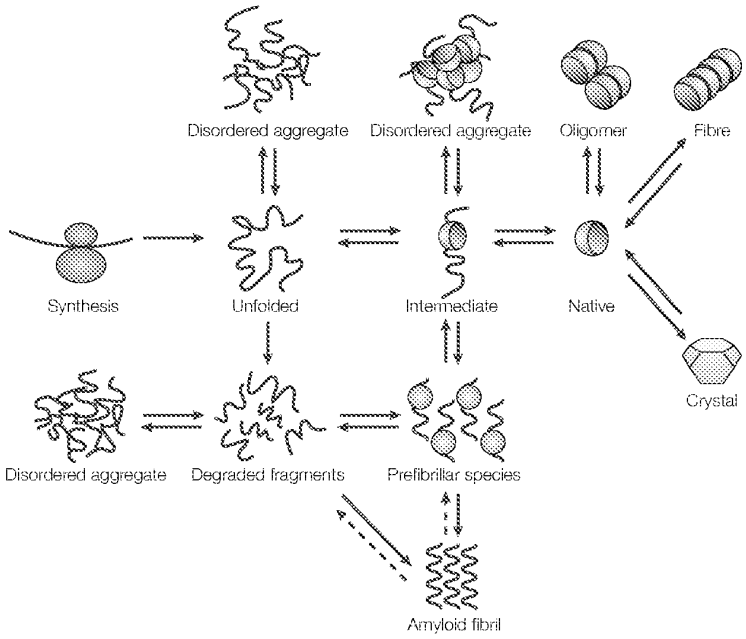


Figure 5 | **The many conformational choices for a polypeptide chain.** Adapted, with permission, from REF. 138 © (2003) Macmillan Magazines Ltd.

to be expected, simply because there has not been evolutionary pressure to evolve one particular kind of fibril, and fibrillogenic proteins are typically flexible, giving access to many conformations. Calorimetric[85] and structural[86] studies also indicate that fibrils are more porous than conventional globular proteins.

Peptides are more of a liability than proteins because of their greater flexibility and access to fibrillogenic conformations. The peptide hormone glucagon, which does not fibrillate in the body, can nevertheless fibrillate fairly easily if mishandled by, for example, prolonged storage at 2.5 mg per ml or higher at 37° C[87]. It is possible to select for less aggregation-prone peptide variants by fusing the peptide in question to green fluorescent protein (GFP), which does not show fluorescence when it accumulates in inclusion bodies[88] and thereby indicates loss of biological structure and function.

Formulating longevity: additives and storage

Recent work has revealed a strong correlation between different types of kinetic stability, as evaluated by unfolding in denaturant conditions and detergents, as well as by proteolysis resistance[89], and has found oligomeric β-sheet proteins to be particularly robust. These proteins are more rigid than their α-helix or mixed α/β counterparts. Analogously, proteins become both rigid and kinetically stable in very high concentrations of organic solvent[90,91], probably because the lack of bulk water acting as a lubricant reduces the energetic driving force for partial and global unfolding. Kinetic stability can be increased by introducing hydrophobic mutations, disulphide bonds, salt bridges and metal ions at the protein surface to stabilize and rigidify regions involved in local unfolding[92].

Because of the grave consequences of protein fibrillation in neurodegenerative diseases, the prevention of aggregation *in vivo* is a subject of intense research[93]. Many of the medically relevant fibrillating proteins have been subjected to extensive screening procedures, resulting in promising small-molecule leads for α-synuclein[94] and tau[95], for example. Fragments of the fibrillating peptide, which can bind to the growing edges of the fibrils by hydrogen bonding but prevent further elongation due to their small size (or by the inclusion of 'β-breaker' residues such as proline), also have an effect[96]. A combination of these two approaches has led to the use of small molecules with hydrogen-bonding patterns complementary to the exposed β-strand, but not to new incoming β-strands[97]. An even more ingenious 'Trojan horse' approach has been to couple the fibril-binding dye to a peptide, which, when in the cell, binds to the FK506-binding protein (FKBP) chaperone, thereby increasing its steric bulk and blocking further fibrillation[98]. Inhibiting aggregation under application-relevant conditions *in vitro* is obviously also very important. Mutagenesis to reduce aggregation is problematic because it can entail extensive clinical trials to document lack of other adverse effects. A more direct approach, therefore, is to alter the formulation conditions of the protein, but this is also challenging. Many therapeutic proteins are required in high doses, administered as part of frequent-dosing regimens, which means that it is important to keep them stably dissolved for extended periods of time at concentrations of tens of mg per ml. Determing the solubility of a protein is a complex task, because of the propensity of proteins to interact with themselves, surfaces and solutes. Of the many techniques used for concentrating proteins, none are without problems[99], and most are only appropriate at certain concentrations of protein.

At present, the prevention of aggregation remains largely empirical, due to a lack of insight into the molecular details of the aggregation process (see REF. 39 for an excellent overview of different approaches). One popular approach is to stabilize the protein and thereby reduce access to partially folded conformations favouring aggregation by hydrophobic contacts, for example. A typical strategy is to add sugars or salts to a protein solution. These solutes are thought to be preferentially excluded from the surface of the protein, therefore favouring a compact state[100,101]. However, because aggregation buries even more surface area per protein molecule, over-stabilization can ultimately lead to aggregation. Other stabilizers include polyols, PEGs and other polymers that sterically hinder protein–protein interactions and limit diffusion. Free amino acids are also often used; arginine is particularly good at preventing aggregation during the refolding of proteins from inclusion bodies[102]. A more sophisticated approach is to add 50 mM each of arginine and glutamate, which leads to a marked increase in the long-term stability of the sample, and also prevents aggregation, precipitation and proteolysis[103]. It has been proposed that the basis for this remarkable effect lies in the capacity of the pair to neutralize opposite charges (which would otherwise lead to intermolecular association) combined with the ability to cover adjoining hydrophobic areas through aliphatic tails[103].

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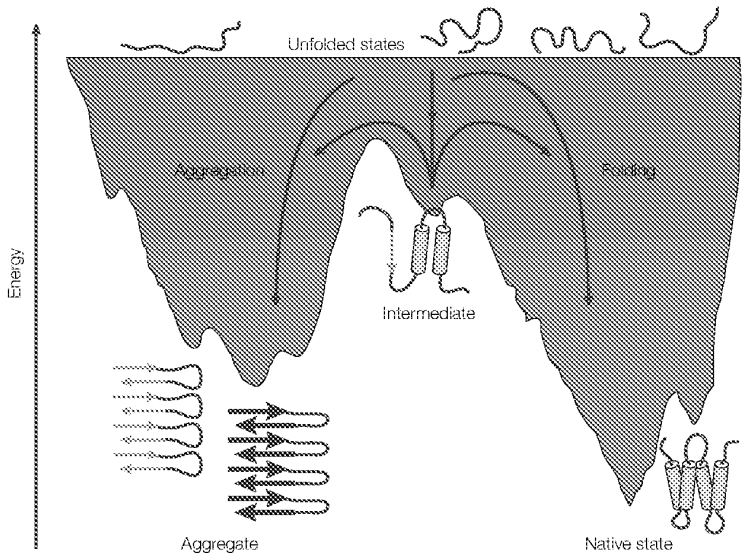


Figure 6 | **The double funnel of folding and aggregation.** Folding is a journey through conformational space, whose architecture is sensitive to environmental conditions, including protein concentration. Whether the protein chooses the folding or aggregation funnel will depend on these conditions. Note that the aggregation funnel is less jagged than the native funnel, emphasizing that there is space for more 'conformational largesse' due to the lower restrictions on aggregate conformations. Adapted, with permission, from REE 66 © (2004) Elsevier Science.

Detergents and other amphiphiles. Non-ionic detergents are often used to reduce the effect of shear, as they outcompete proteins at the air–water interface[104], but this can sometimes be counterbalanced by accelerated aggregation under long-term motionless shelf storage[105]. They are sometimes[106], but not always[107], able to prevent aggregation induced by heat. Ionic surfactants are generally denaturing, but reversible unfolding has been used to prevent aggregation of heat-denatured RNase[108]. They tend to stabilize α-helical conformations that do not form intermolecular contacts with the same alacrity as β-strands. However, just as the water–chloroform interface can be a powerful stimulant of aggregation[45], recent work indicates that anionic surfaces encourage protein aggregation and fibrillation — for example, through negatively charged phospholipid vesicles[109,110], anionic surfactants[111] and carboxylate-modified polystyrene beads[112]. The phospholipid environment encountered *in vivo* is chemically diverse, providing an opportunity for both hydrophobic interactions in the transmembrane region as well as polar and electrostatic contacts through the head group of lipids in the membrane. It has been suggested that the lipid surface acts to concentrate and align protein molecules, possibly through clusters of cationic residues on the protein. Another mechanism could be for the negative surface to stabilize a fibrillogenic monomer conformation, which through intermolecular interactions forms the nucleus for subsequent fibrillation[112].

Cyclodextrins. A class of substances that has been found to have significant potential in reducing aggregation is the cyclodextrins. These are circular polymers of typically five to seven glucose rings[113], although larger polymers also occur[114]. Cyclodextrins suppress aggregation of therapeutically relevant proteins, such as insulin[115–117] and growth hormone[118,119], as well as several other proteins[120,121]. This derives from their ability to bind to aromatic residues[122], which can lead to preferential stabilization of the unfolded state[123] and a reduction in folding rates[124]. The ability to suppress aggregation has also led to their use as simple chaperones for the refolding of proteins in combination with SDS[107,125]. The commonly used β-cyclodextrin is not approved for human consumption in an unmodified form because it is capable of extracting biomembrane components[126], and forms a highly insoluble complex with cholesterol, which could lead to toxic effects after prolonged exposure[127]. However, careful derivatization of β-cyclodextrin alters its selectivity towards biomembrane components and minimizes its toxicity[127]. Drug formulations containing hydroxypropyl-β-cyclodextrin (for the peptides leucine enkephelin and a neuromedin B-receptor antagonist) and sulphobutylether-β-cyclodextrin (for the small-molecule drugs ziprasidone (Geodon; Pfizer) and voriconazole (Vfend; Pfizer) have been approved for parenteral drug administration.

Lyophilization. Storing proteins in solution at low temperatures generally extends their shelf-life. Proteins can be denatured at low temperatures in the liquid phase, but, in general, cold denaturation is reversible, unlike most high-temperature denaturation, and so offers no practical problems[128]. Freezing obviously provides access to even lower temperatures, but repeated cycles of freeze–thawing can strongly stimulate aggregation by providing nucleation surfaces at the ice–water interfaces[105], as well as leading to pH changes and phase separation[129]. Another approach is therefore to restrict protein mobility by lyophilization or freeze-drying, for example. Freeze-drying is the separation of liquid water from a solution frozen to ice by vacuum sublimation, leaving the solutes in an anhydrous or almost anhydrous state. As described by Franks[130,131], the process is not just trial-and-error, but can be controlled by well-established physicochemical and engineering principles, taking into account parameters such as the composition and concentration of the product, the type of container, the equipment and process cycle (including primary and secondary drying). The method is not without challenges, because differential precipitation of buffers or other solvent compositions during freezing can lead to pH changes that irreversibly inactivate proteins. This can be counteracted by excipients, such as certain carbohydrates, soluble polymers, salts and volatile compounds, which form glasses during freezing, which in effect preserves liquid properties in the solid state and prevents unwanted crystallization and chemical side reactions. However, their effect on the glassing temperature must also be taken into account. Temperature and chamber pressure both affect sublimation rates and can be combined judiciously to avoid overheating and structural collapse of the lyophilized solute network. Assessing the stability of the freeze-dried product is not straightforward; accelerated storage

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testing by product stressing can be misleading, because the Arrhenius kinetics used to extrapolate to low temperatures are not appropriate for temperatures around the glass transition[132]. It should also be noted that proteins can undergo reversible conformational changes in the lyophilized state that expose otherwise buried regions, making them susceptible to undesirable side reactions, such as disulphide bond shuffling in the presence of trace moisture. Examples include insulin[133] and β-galactosidase[134] (see also REF. 135 and references therein). Other crosslinking reactions include insulin transamidation[136] and dityrosine formation occurring under oxidative conditions or ultraviolet stress[137].

Conclusions

The successful development of protein-based medicines depends on an intimate understanding of their physico-chemical and biological characteristics, including chemical and physical stability, and their efficacy and safety profile. To get new, safe therapeutic proteins to the market more quickly, it is important to obtain a detailed understanding of what kind of protein modification might be acceptable from a safety and efficacy point of view early in the development process. However, it is equally important to understand the mechanisms by which the protein structure can be compromised during bulk processing and production of the final product. This includes strategies to reduce or prevent chemical degradation, denaturation, aggregation and other structural changes that could prove to be prohibitive for successful development of the drug. It is the joint responsibility of academia, the pharmaceutical industry and the regulatory authorities to establish the scientific background for the safe, fast testing and assessment of promising new biopharmaceuticals to the benefit of patients and society.

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Competing interests statement

The authors declare no competing financial interests.

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The following terms in this article are linked online to:

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FoldX — a force field for energy calculations in proteins:
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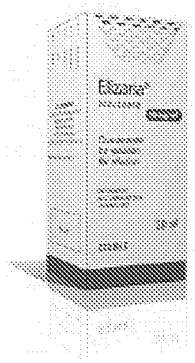
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Final Report

Antibody Full Length Sequencing of Hybridoma 5G1.1 T175

Order Number: U856UEK140-1

November 15, 2019

Prepared by

Jinquan Chen

Jinquan Chen, Production Supervisor

This study was conducted by strictly following the procedures described in this report. All data presented are authentic and accurate to the best of our knowledge. Information and materials provided by this investigation are for research use only.

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Recommended Analysis Tools

We recommend the following free online tools for sequence analysis of immunoglobulin variable regions:

i. NCBI Nucleotide BLAST
ii. IMGT/V Quest program
iii. NCBI IgBLAST

Antibody sequences of 5G1.1 T175 were listed as below:

Heavy chain: DNA sequence (1416 bp)

Signal sequence-FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4-Constant region-Stop codon

ATGGAATGGACCTGGGTCTTTCTCTTCCTCCTGTCAGTAACTGCAGGTGTCCACTCCCAGGTTCAGC
TGCAGCAGTCTGGAGCTGAGCTGATGAAGCCTGGGGCCTCAGTGAAGATGTCCTGCAAGGCTACTGG
CTACATATTCAGTAACTACTGGATACAGTGGATAAAGCAGAGGCCTGGACATGGCCTTGAGTGGATT
GGTGAGATTTTACCTGGAAGTGGTTCTACTGAGTACACTGAGAACTTCAAGGACAAGGCCGCATTCA
CTGCAGATACATCCTCCAACACAGCCTACATGCAACTCAGCAGCCTGACATCAGAGGACTCTGCCGT
CTATTACTGTGCAAGATATTTCTTCGGTAGTAGCCCCAACTGGTACTTCGATGTCTGGGGCGCAGGG
ACCACGGTCACCGTCTCCTCAGCCAAAACAACAGCCCCATCGGTCTATCCACTGGCCCCTGTGTGTG
GAGATACAACTGGCTCCTCGGTGACTCTAGGATGCCTGGTCAAGGGTTATTTCCCTGAGCCAGTGAC
CTTGACCTGGAACTCTGGATCCCTGTCCAGTGGTGTGCACACCTTCCCAGCTGTCCTGCAGTCTGAC
CTCTACACCCTCAGCAGCTCAGTGACTGTAACCTCGAGCACCTGGCCCAGCCAGTCCATCACCTGCA
ATGTGGCCCACCCGGCAAGCAGCACCAAGGTGGACAAGAAAATTGAGCCCAGAGGGCCCACAATCAA
GCCCTGTCCTCCATGCAAATGCCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTCATCTTCCCT
CCAAAGATCAAGGATGTACTCATGATCTCCCTGAGCCCCATAGTCACATGTGTGGTGGTGGATGTGA
GCGAGGATGACCCAGATGTCCAGATCAGCTGGTTTGTGAACAACGTGGAAGTACACACAGCTCAGAC
ACAAACCCATAGAGAGGATTACAACAGTACTCTCCGGGTGGTCAGTGCCCTCCCCATCCAGCACCAG
GACTGGATGAGTGGCAAGGAGTTCAAATGCAAGGTCAACAACAAAGACCTCCCAGCGCCCATCGAGA
GAACCATCTCAAAACCCAAAGGGTCAGTAAGAGCTCCACAGGTATATGTCTTGCCTCCACCAGAAGA
AGAGATGACTAAGAAACAGGTCACTCTGACCTGCATGGTCACAGACTTCATGCCTGAAGACATTTAC
GTGGAGTGGACCAACAACGGGAAAACAGAGCTAAACTACAAGAACACTGAACCAGTCCTGGACTCTG
ATGGTTCTTACTTCATGTACAGCAAGCTGAGAGTGGAAAAGAAGAACTGGGTGGAAAGAAATAGCTA
CTCCTGTTCAGTGGTCCACGAGGGTCTGCACAATCACCACACGACTAAGAGCTTCTCCCGGACTCCG
GGTAAATGA

Heavy chain: Amino acid sequence (471 aa)

Signal peptide-FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4-Constant region-Stop codon

MEWTWVFLFLLSVTAGVHSQVQLQQSGAELMKPGASVKMSCKATGYIFSNYWIQWIKQRPGHGLEWI

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GEILPGSGSTEYTENFKDKAAFTADTSSNTAYMQLSSLTSEDSAVYYCARYFFGSSPNWYFDVWGAG
TTVTVSSAKTTAPSVYPLAPVCGDTTGSSVTLGCLVKGYFPEPVTLTWNSGSLSSGVHTFPAVLQSD
LYTLSSSVTVTSSTWPSQSITCNVAHPASSTKVDKKIEPRGPTIKPCPPCKCPAPNLLGGPSVFIFP
PKIKDVLMISLSPIVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTLRVVSALPIQHQ
DWMSGKEFKCKVNNKDLPAPIERTISKPKGSVRAPQVYVLPPPEEEMTKKQVTLTCMVTDFMPEDIY
VEWTNNGKTELNYKNTEPVLDSDGSYFMYSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTP
GK*

Light chain: DNA sequence (702 bp)

Signal sequence-FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4-Constant region-Stop codon

ATGGGTGTACCCACTCAGCTCCTGTTGCTGTGGCTTACAGTCGTAATTGTCAGATGTGACATCCAGA
TGACTCAGTCTCCAGCTTCACTGTCTGCATCTGTGGGAGAAACTGTCACCATCACATGTGGAGCAAG
TGAGAATATTTACGGTGCTTTAAATTGGTATCAGCGGAAACAGGGAAAATCTCCTCAGCTCCTGATC
TATGGTGCAACCAACTTGGCAGATGGCATGTCATCGAGGTTCAGTGGCAGTGGATCTGGTAGACAGT
ATTATCTCAAGATCAGTAGCCTGCATCCTGACGATGTTGCAACGTATTACTGTCAAAATGTGTTAAA
TACTCCTCTCACGTTCGGTGCTGGGACCAAGTTGGAGCTGAAACGGGCTGATGCTGCACCAACTGTA
TCCATCTTCCCACCATCCAGTGAGCAGTTAACATCTGGAGGTGCCTCAGTCGTGTGCTTCTTGAACA
ACTTCTACCCCAAAGACATCAATGTCAAGTGGAAGATTGATGGCAGTGAACGACAAAATGGCGTCCT
GAACAGTTGGACTGATCAGGACAGCAAAGACAGCACCTACAGCATGAGCAGCACCCTCACGTTGACC
AAGGACGAGTATGAACGACATAACAGCTATACCTGTGAGGCCACTCACAAGACATCAACTTCACCCA
TTGTCAAGAGCTTCAACAGGAATGAGTGTTAG

Light chain: Amino acid sequence (233 aa)

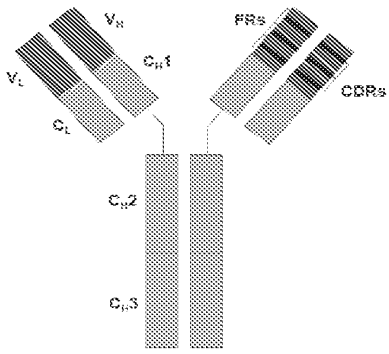
Signal peptide-FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4-Constant region-Stop codon

MGVPTQLLLLWLTVVIVRCDIQMTQSPASLSASVGETVTITCGASENIYGALNWYQRKQGKSPQLLI
YGATNLADGMSSRFSGSGSGRQYYLKISSLHPDDVATYYCQNVLNTPLTFGAGTKLELKRADAAPTV
SIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTYSMSSTLTLT
KDEYERHNSYTCEATHKTSTSPIVKSFNRNEC*

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Sequence	Clones sequenced	Clones with >99% sequence identity
V_H	5	5
C_H	5	5
V_L	5	5
C_L	5	5



IMGT Analysis of V(D)J Junctions

Sequence	V-GENE and allele	Functionality	V-REGION identity % (nt)	J-GENE and allele	D-GENE and allele	AA Junction	Junction frame
V_H	Musmus IGHV1-9*01 F	productive	93.06% (268/288 nt)	Musmus IGHJ1*01 F	Musmus IGHD1-1*01 F	CARYFF GSSPNW YFDVW	in-frame
V_L	Musmus IGKV12-89*01 F	productive	99.28% (277/279 nt)	Musmus IGKJ5*01 F		CQNVLN TPLTF	in-frame

Isotype of antibody

Isotype is mouse IgG2a/kappa, as analyzed by the sequences of constant region.

Materials

Hybridoma cells provided by the client;
TRIzol® Reagent (Ambion, Cat. No. : 15596-026);
PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara, Cat. No. : 6110A).

Methods

Total RNA was isolated from the hybridoma cells following the technical manual of TRIzol® Reagent. Total RNA was then reverse-transcribed into cDNA using either isotype-specific anti-sense primers or universal primers following the technical manual of PrimeScript™ 1st Strand cDNA Synthesis Kit. Antibody fragments of heavy chain and light chain were amplified according to the standard operating procedure (SOP) of rapid amplification of cDNA ends (RACE) of GenScript. Amplified antibody fragments were cloned into a standard cloning vector separately. Colony PCR was performed to screen for clones with inserts of correct sizes. The consensus sequence was provided.

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Part 1. mAb sequencing: Samples summary

1.1 Sample list

NO	Order	Quote	Cell Line	V/F	Species	Isotype	Remarks
1	U856UEK140-1	Q1616250	5G1.1 T175	F	Mouse	NA	Emergency Order

1.2 No 1 (total of 1 vials) were received. Sample status Frozen

Recipient Yuling Li Date 11/13/2019

Part 2. mAb sequencing: RNA extraction and reverse transcription

2.1 Reagents and lot#

Name	LOT#
AxypreP multisource total RNA miniprep kit	160035113
PrimeScript™ 1st strand cDNA Synthesis Kit	1803957A
ddH_2O	10/12/2019

2.2 RNA samples summary and reverse transcription reaction setup

NO.	Order	Volume (μL)	RNA Concentration (ng/μL)	$OD_{260/280(230)}$	Reverse primer (μL)	RNA(μL)	H2O(μL)
1	U856UEK140-1	20	1070.2	2.11/1.02	CDSIII	1	3

No 1 samples total RNA were extracted. Total of 1 samples were performed with reverse transcription.

2.3 RNA electrophoresis

The results of RNA electrophoresis are kept at the following address:

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Operator Yuling Li Date 11/13/2019

The isolated total RNA samples were run alongside a DNA marker Marker III (TIANGEN, Cat. No. : MD103) on a 1.5% agarose/GelRedTM gel (Fig. 1).

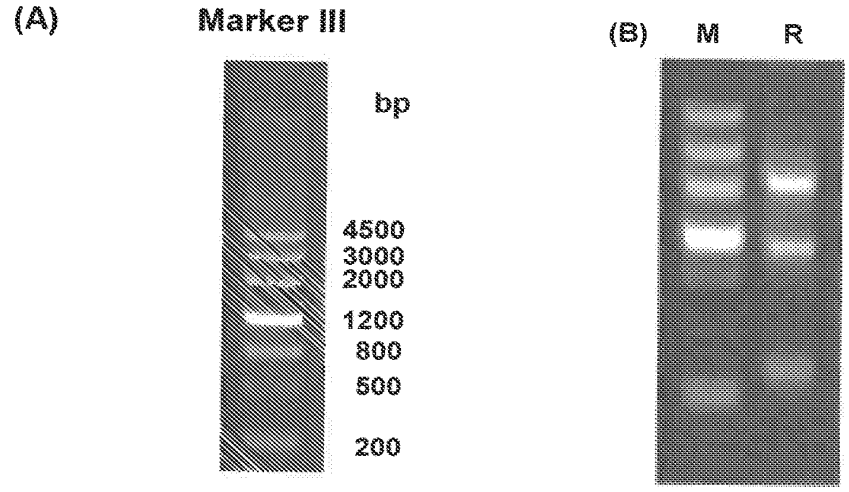


Fig. 1. Agarose gel electrophoresis of total RNA from the provided hybridoma cells of U856UEK140-1. (A) DNA marker Marker III. (B) Lane M, DNA marker Marker III; Lane R, Total RNA from the provided hybridoma cells.

Part 3. mAb sequencing: PCR amplification & TA cloning

3.1 Reagents & lot#

Name	LOT#
10X pfu buffer	18H13
Pfu polymerase	AIBG
dNTP	C70011903
ddH_2O	10/12/2019
AxyPrep Gel DNA extraction kit 250-prep	08119KE1
5xTA /Blunt-Zero Cloning Mix	10/24/2019

3.2 PCR setup

2ul of DNA template (cDNA) in 50ul reaction volume

PCR and sequencing results of the provided cells

No.	Gene	RT primer	5' primer	3' primer	PCR results	Sequencing results
1	β-actin	CDSIII	Actin F_1	Actin R_1	Positive	-
2	VH-G	CDSIII	Race-F	mG275	Positive	Productive heavy chain variable region
3	VH-M	CDSIII	Race-F	mM230	Non band	
4	VH-A	CDSIII	Race-F	mA262	Non band	
5	VK	CDSIII	Race-F	mK232	Positive	Productive Light chain variable region
6	VL	CDSIII	Race-F	mL281	Band is low and weak	Non antibody sequence
7	CH-G	CDSIII	3'primer	mG16	Positive	Productive heavy chain constant region
8	CH-M	CDSIII	3'primer	mMCHF1	Non band	
9	CK	CDSIII	3'primer	mK20	Positive	Productive Light chain constant region
10	CL	CDSIII	3'primer	mL32	Positive	Non antibody sequence

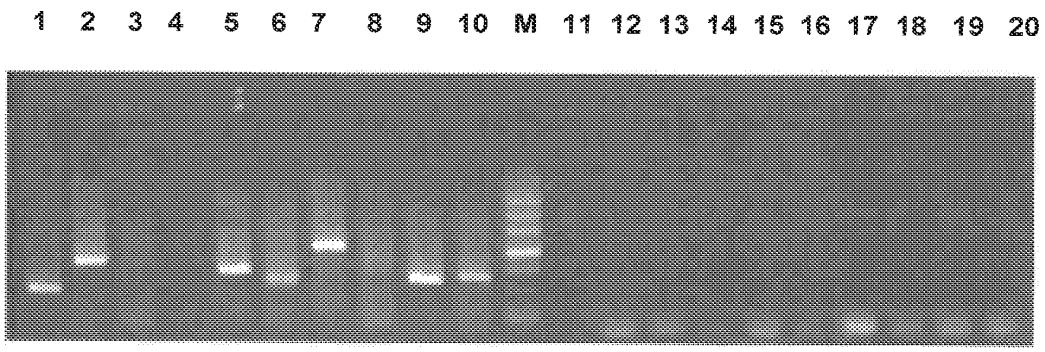


Fig. 2. Variable genes amplification from the provided cells. Lane M, DNA Marker III; Lane 1 to Lane 20, corresponding to No. 1 to No. 10 in Table 2; Lane 11 to Lane 20, corresponding to CTL in Table 2; (CTL-: Negative control.)

3.3 DNA electrophoresis

The results of DNA electrophoresis are kept at the following address:

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3.4 Gel extraction

Total of 4 gel slices were recovered. DNA was extracted and eluted with 30 ul of elution buffer.

3.5 Connection and transformation

Total of 4 DNA fragments were cloned ligated and cloned into pCE2-TA vector.

Operator Yuling Li Date 11/13/2019

3.6 Colony screening

3ul of template (cell culture) in 30ul reaction volume

No.1	Template	Primers
VH and VK	cell culture	M13-47 M13-48 P541 DMSO

The results of Colony screening are kept at the following address:

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Operator Yuling Li Date 11/4/2019

Part 4. mAb sequencing: Analysis of sequencing results

4.1 Sequencing results and analysis

NO.	Order	VH	VK	CH	CK	QC
1	U856UEK140-1	5	5	5	5	Pass

The detail analysis of sequencing results are kept at the following address:

原 30\内部\2 各流程文件\12 AM 测序组\1 mAb sequencing\ U856UEK140

Operator Yuling Li Date 11/15/2019

Project Proposal for Monoclonal Antibody Sequencing Service

Confidential

Prepared by: GenScript

GenScript Inc.

Date: Nov 5th, 2019

Note:

This proposal containing study design, price estimate and any attached files is the properties of GenScript USA Inc. and contains confidential and privileged information that is purposed for the only use of the sponsor. Any review, copying, or distribution of this study quotation or any attachments for other than its purposed use is not authorized and may be unlawful.

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Project Description

GenScript will provide the client with the service of monoclonal antibody sequencing from hybridoma cells. The deliverables are DNA sequences of full length antibody of hybridoma.

1. Materials

To be ordered and prepared by Client /GenScript

Materials	Provider	Quality and quantity
Frozen hybridoma cells with anti-freezer	Client	10^6 cells, 2 vials for each cell line;
The species information of each sample	Client	N/A

2. Project Procedure (Standard –full length sequencing) (SC1919)

a. Extract total RNA from the provided hybridoma cells. RNA quality assessment.

Evaluate the quality of RNA samples by gel electrophoresis and determine the RNA concentration by NanoDrop2000C.

b. Reverse transcription to cDNA and PCR amplification of variable and constant regions

Perform reverse transcription according to the manufacturer's protocol of PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara). Run 5' RACE PCR according to GenScript's standard operation procedure to amplify the variable and constant region genes of heavy chain and light chain, respectively.

Gel purify the target PCR fragments (~500 bp) and clone the fragments into sequencing vector, respectively.

c. Colony PCR screening and sequencing

Perform colony screening and select 5 independent positive clones each for subsequent DNA sequencing.

d. Sequence analysis

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Sequence analysis with V region germline database (IMGT).

Deduce the consensus sequence of VH and VL for each monoclonal hybridoma cell line.

Prepare final report and delivery.

3. Final deliverables

GenScript will provide to the client the following materials upon completion of the project:

a. Report with leader sequencing, constant region service and CDR identification.

b. Alignments of the heavy and light chains

4. Price and timeline summary

Item	Unit	Price ($)	Time
Full length sequencing (SC1919) (additional charge for unknown isotope)	1	2,850	5 business Day
Rush processing fee	1	2,500	N/A

5. Terms and Termination

If at any point of the project, Client and GenScript both agree that the project should end, the project will end, and GenScript will charge the client by the work that is already finished. Terms, such as price and timeline defined in this document are subject to change.

Confidential

Complementarity, specificity and the nature of epitopes and paratopes in multivalent interactions

Neil S. Greenspan and Laurence J.N. Cooper

Structural elements of an antibody (Ab) or antigen (Ag) distant from the actual sites mediating contact between Ab and Ag can exert substantial influence on binding to, and discrimination among, multivalent targets. Consequently, multivalent molecules that express the same number of identical binding sites, but that differ in other structural features, can exhibit differences in their ability to discriminate between multivalent ligands. Here, Neil Greenspan and Laurence Cooper review evidence for these effects, and explore implications of the conclusion that effective specificity in multivalent interactions is not completely determined by the degree of complementarity between epitopes and paratopes.

Specificity is a fundamental but multifaceted immunological concept. For instance, Eugene Day identifies three major immunological meanings for the term[1]. First, specificity can be viewed as a measure of the 'goodness of fit' between the antibody (Ab) combining site (paratope) and the antigenic determinant (epitope). Second, specificity can refer to the extent of discrimination between two, or more, monovalent antigens (Ags) mediated by a given Ab. Third, specificity can be used in the context of discrimination between two, or more, multivalent Ags. A fourth sense of specificity may be worthy of consideration, based on observations that the extent of binding of Ab to Ag is not necessarily directly related to the magnitude of the biological effect. For example, comparable amounts of two different Abs, of the same isotype, bound to a cell surface do not always result in comparable amounts of complement activation[2]. Such effects imply that biological specificity can diverge from immunochemical specificity. It is also important to note that biologically relevant discrimination can result in some cases from differences in kinetic constants, as has been suggested for B-cell activation[3], and not only from differences in equilibrium constants.

This article discusses the limitations of the concept of molecular complementarity, with particular reference to data that have bearing on the structural basis for specificity as conceived in the third of the meanings described above. Although the focus of discussion is on discrimination among multivalent Ags by Abs, the chief conclusions may be of broader relevance.

Complementarity and monovalent interactions

Paul Ehrlich's studies on Ab–Ag interactions, together with Emil Fischer's studies on enzyme–substrate interactions, spurred initial development of the notion that two molecules would be likely to bind to one another if they shared complementary surfaces[4]. Linus Pauling, the primary developer of the modern concept of molecular complementarity (or 'complementariness' as described by Pauling), effectively promulgated the notion that it is the degree of complementarity between sites on ligand and receptor that determines the affinity and specificity of the ligand–receptor interaction[5]. Given that this notion has received virtual universal acceptance, it is of interest to note that the degree of complementarity (as operationally defined) between a receptor and a ligand is not always directly related to the free energy of complex formation (or intrinsic affinity)[6]. Similarly, it is difficult to find data documenting that the differential in the free energy of complex formation, for pairings of a receptor with two different ligands, is directly related to the corresponding difference in complementarity (i.e. specificity according to the second of the meanings above). One reason for the failure to document such relationships is that there is no unique method for determining the magnitude of complementarity for a bimolecular complex, even with the availability of a high-resolution crystallographic structure of the complex[7]. Second, complementarity, as determined by any particular scheme of calculation, fails to take full account of one or more of the following factors contributing to the energetics of intermolecular interactions[8]: (1) the energetic effects of rearrangements of solvent molecules and counterions, which remain difficult to calculate[9,10]; (2) the direct contributions of bound water molecules and ions[11]; (3) the conformational alterations of either receptor or ligand associated with complex formation[12]; and (4) the charge-dependent[13] and charge-independent[14] long-range effects. The following discussion will examine data from our own studies and from those of others, which suggest that different receptors with two or more identical binding sites (identical complementarity) do not necessarily bind equivalently to, or express identical specificity for, multivalent targets.

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IgG subclass-related discrimination among multivalent Ags

A set of monoclonal Abs (mAbs), known as HGAC 39, with identical variable (V) domains specific for the *N*-acetylglucosamine (GlcNAc) residues of streptococcal group A carbohydrate, the cell-wall polysaccharide of *Streptococcus pyogenes*, have been examined with respect to binding properties. These mAbs, representing the mouse IgG1, IgG2b and IgG3 subclasses, were compared in binding assays both with group A streptococci and with two bacterial strains derived from group A streptococci, as well as with conjugates of GlcNAc covalently coupled to bovine serum albumin (BSA). The strains of streptococci differed in the density of surface GlcNAc residues, and will be referred to as high-, intermediate- and low-epitope-density bacteria. Similarly, the GlcNAc–BSA conjugates differed in GlcNAc-substitution ratio (denoted as $GlcNAc_x$, where x is the substitution number).

Earlier studies established that the IgG3 mAb can bind to multivalent GlcNAc arrays by an Fc-region-dependent cooperative mechanism under conditions where the IgG1 and IgG2b mAbs do not exhibit detectable cooperativity[15–18]. It was also demonstrated that: (1) the IgG3 mAb binds more effectively to the various strains of streptococci, as well as to commercially synthesized $GlcNAc_{16}$–BSA, than the IgG1 and IgG2b mAbs or the IgG3-derived $F(ab')_2$ fragments; and (2) increased amounts of soluble GlcNAc were required to achieve 50% inhibition of the binding of the IgG3 mAb than were required to mediate 50% inhibition of the binding of the IgG1 and IgG2b mAbs or IgG3-derived $F(ab')_2$ fragments. Thus, it was established that differences in the heavy chain constant (C_H) domains, and in the Fc region in particular, substantially influence the apparent functional affinities (avidities) of these IgG mAbs (Ref. 18). In particular, the superior binding of the IgG3 mAb, relative to the IgG1 and IgG2b mAbs, was presumed to stem (at least partially) from the ability of IgG3 mAbs to engage in non-covalent association (IgG3–IgG3), mediated by sites in the Fc region.

Further studies focused on the relative binding patterns of the mAbs and $F(ab')_2$ fragments to bacterial or glycoconjugate Ags displaying different epitope densities. It was observed that the mAbs exhibited different patterns of binding[19,20]; in other words, they differed in their ability to discriminate between the various multivalent Ag targets. For instance, we carried out flow cytometric experiments with the high-, intermediate- and low-epitope-density streptococci in which the different bacteria were mixed with the appropriate Ab species at varying concentrations. Unbound GlcNAc-specific Ab was washed away and, following fixation, the remaining bound Ab was quantitated with fluorescein isothiocyanate (FITC)-conjugated goat Ab specific for mouse Cκ domains. In this assay, the IgG3 mAb bound best to the intermediate-epitope-density strain, while the IgG1 and IgG2b mAbs, and the IgG3-derived $F(ab')_2$ fragments, bound best to the high-epitope-density strain. These results suggest that: (1) the immunochemical identity of multivalent Ags can depend both on epitope organization, or spacing, and on epitope fine structure; (2) for antigenic targets differing in average epitope spacing, the discriminatory abilities of Abs can be influenced by structural differences in the Fc region or other sites residing in the C_H domains. Thus, these results imply that the paratope, as traditionally defined, does not comprise all of the amino acid residues that determine relative binding to different antigenic targets. In this instance, we attribute at least part of the C_H-domain effect on apparent specificity to an influence on the extent of cooperative (noncovalent) interactions between IgG molecules. However, a contribution from structural features that influence segmental flexibility cannot be discounted.

Although, at present, we cannot implicate differences in segmental flexibility as contributing to the subclass-related differences in binding among the GlcNAc-specific mouse mAbs, it has been speculated that such flexibility is crucial for maximizing monogamous, bivalent binding to multivalent targets[21,22]. An implicit consequence of this notion is that differences in segmental flexibility could contribute to differences in relative binding (i.e. specificity according to the third of the meanings described above) for multivalent Ags that vary in epitope density. We are not aware of any systematic study that has addressed this speculation with regard to mouse IgG Abs. However, two recent reports[23,24] have demonstrated differences in binding to multivalent Ags for Abs expressing identical V domains but different human C_H domains. In both cases, the investigators suggested that differences in hinge region structure and segmental flexibility contribute to the binding differences.

The above results suggest that self–nonself discrimination in the effector phase of humoral immunity can depend on the spatial distribution of epitopes, as well as on the fine structure of the epitopes. Indeed, a recent study suggests that differences in the spacing or distribution of a protein epitope on vesicular stomatitis virus (VSV) glycoprotein, displayed on virions as opposed to infected cells, can influence the extent of triggering of cognate B cells by these two immunogens[25]. This result parallels our observations relating to the efferent phase of the immune response, by suggesting that self–nonself discrimination in the afferent phase of the humoral immune response can depend both on epitope distribution and on epitope fine structure.

Specificity and DNA-binding proteins

Several studies of transcription factors have suggested that biochemical and biological specificity for DNA can be influenced by changes in the spacing between the nucleotide sequences physically contacted by protein, as well as by changes in the sequences of the actual contact sites[26,27]. A study of particular importance to this concept is that of Cuenoud and Schepartz, who examined several constructs containing so-called bZIP DNA-recognition domains[28]. These domains contain basic amino acids and are found in proteins containing leucine-zipper dimerization motifs. In this study, the authors examined constructs containing two identical bZIP domains linked through either a leucine-zipper peptide, a disulfide bond or a chelation complex of iron [bis(terpyridyl)iron(II)]. It was found that the molecule

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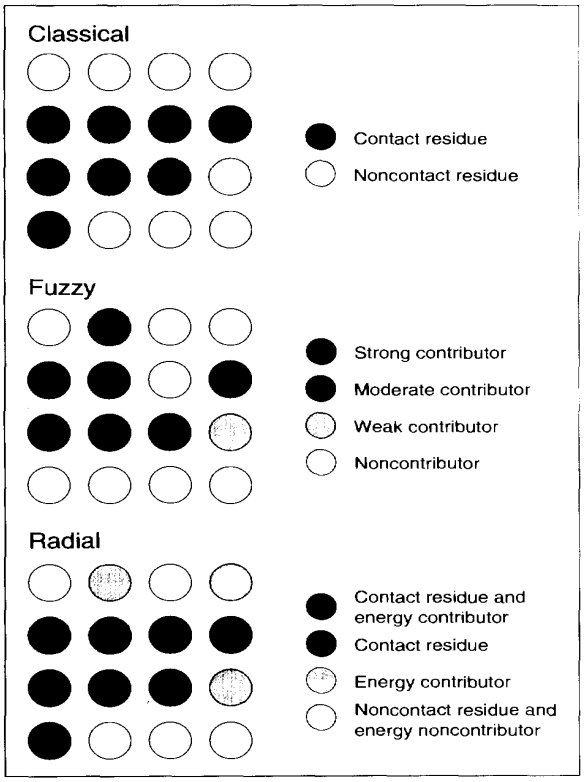


Fig. 1. *Schematic illustration of three different conceptualizations of a paratope as a set of amino acid residues or, alternatively, as a set of atoms. Each circle represents an amino acid residue (atom) in a simplified, hypothetical antibody (Ab). The top and bottom rows correspond to variable module framework residues or amino acid residues in the constant domains, and the middle two rows correspond to hypervariable residues. In the case of the 'classical paratope set', amino acid residues are either part of the paratope or not, depending only on whether the residue in question participates in contacting a given antigen (Ag). Amino acid residues belong to the 'fuzzy paratope set' to different degrees, as indicated by the intensity of the color of each circle. In this case, the degree of membership in the paratope set is correlated with the extent to which each residue contributes to the free energy of complex formation. A similar approach could be based on other variables characterizing the complex, such as the extent of contribution to buried surface area. If the paratope is conceived of as a 'radial' category, then elements of the paratope set include, in this simplified scheme, amino acid residues that contribute to the free energy of complex formation (i.e. stabilize the complex) or that mediate contact between Ab and Ag. Some residues contribute in both senses, others contribute in only one sense or the other. The most realistic concept of a paratope set might include variable degrees of set membership, as well as permit set membership on the basis of only a subset of relevant properties.*

containing the iron complex exhibited strikingly different ratios of binding to cognate and noncognate DNA sites, in comparison with the molecules containing the leucine-zipper motif or the disulfide bond. The cognate sequence differed from the noncognate sequence by the presence of an additional base pair between the two bZIP half-sites.

Thus, in the investigation by Cuenoud and Schepartz, specificity (i.e. discrimination between two different DNA sites) was found to depend on structural features distant from the amino acids that physically contact the DNA, which is analogous to the results with GlcNAc-specific mAbs described above. As the authors state, 'The bis(terpyridyl)iron(II) dimerization domain modifies the inherent selectivity of the GCN4 basic domain without altering its sequence.' Conformational differences in the basic DNA-contacting domains among the three constructs were not formally ruled out in this study. However, these domains are relatively disordered prior to binding to DNA, and the bis(terpyridyl)iron(II)-containing molecule exhibited an increase in α-helical structure typical of bZIP recognition domains upon interaction with cognate DNA. Furthermore, the leucine-zipper-containing and iron-containing molecules gave identical DNase I footprints on cognate DNA, indicating that the DNA-contacting domains for both molecules probably interacted with the DNA in the same manner.

The chief conclusion from the studies described above is that immunological specificity, and molecular specificity in general, can depend on structural features distant from the binding sites (e.g. Ab paratopes) that directly participate in the formation of a bimolecular interface. Since many investigators implicitly regard paratopes and epitopes as the structural correlates of affinity and specificity, it is important to be aware of the potential nonidentity of a site that is defined in terms of intermolecular contact and one that is defined in terms of the structural determinants of affinity or specificity.

While it might seem natural to place primary emphasis on contact residues, each unit of stabilization energy (for complex formation) is equivalent from a thermodynamic perspective, regardless of whether it depends on the identities of amino acids in the contact-mediating regions or in the non-contact-mediating regions of the molecule. As Takeda *et al.*[29] state in a study of the thermodynamics of the interaction between Cro protein and DNA, 'To understand how protein recognizes specific DNA sequences, ..., we must understand the energetics of DNA binding along with the structure because the specificity of DNA association is ultimately determined by the free energy.'

In the context of this last point, an informative recent study examined the thermodynamics of the interaction between *Xenopus laevis* transcription factor IIIA (TFIIIA) and the cognate DNA site from the 5S rRNA gene[30]. TFIIIA contains nine zinc-finger motifs. The authors investigated the thermodynamic consequences of consecutively inactivating, through single amino acid substitutions, each of these nine domains. Measurement of sensitivity to proteolysis of the nine mutant proteins, in comparison with wild-type protein, indicated that the individual fingers were independent structural units. Footprinting by DNase I sensitivity also suggested that the individual zinc-finger domains were largely independent in mediating sequence-specific recognition of DNA. Yet, the binding measurements revealed lower overall affinity of binding for the

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intact protein than predicted from the results with the mutants, implying that there were strong, negative (positive change in free energy) thermodynamic interactions among the zinc-finger domains. Current models of the TFIIIA–DNA interaction, based on structural determinations of other zinc-finger proteins, do not provide a ready mechanistic explanation for these results.

Summarizing the above findings with TFIIIA, substitutions for amino acid residues that participate in one interface in a multivalent interaction may exert substantial thermodynamic effects on other contact sites, even when the tertiary structures of the relevant domains are largely independent. A plausible mechanistic explanation for such negative interactions, as exemplified by TFIIIA, is that simultaneous binding to DNA by all nine zinc-finger sites requires the TFIIIA protein to adopt an energetically unfavorable conformation. Thus, it is not necessary to postulate the transmission of a change in tertiary structure from one zinc finger to another in order to account for thermodynamic coupling between recognition sites[31].

Topes and sets

These last points may be restated in terms of set theory. The standard view of paratopes can be formulated in such terms by asserting that a paratope is a set of amino acids or, at a finer level of resolution, a set of atoms. In classical set theory, all the members of a set share a well-defined list of properties, and membership in a set is either 100% or 0%. Most immunologists would probably draw the borders for inclusion of an amino acid residue in a 'paratope set' at the boundaries between the hypervariable regions and the framework regions, or at the boundary between the V and C regions (Fig. 1).

While acknowledging the value of this view, we suggest that paratopes (or epitopes), particularly in the case of multivalent interactions, can be conceived of as sets with imprecise boundaries or membership requirements. This notion of paratopes may bear some analogy to two types of nonclassical sets: radial categories[32] and 'fuzzy sets'[33] (Fig. 1). A radial category is a class of entities with a central or prototype member(s) that possesses all of the attributes that define the category. Other members of the category can share different subsets of such attributes with the prototype(s). A 'fuzzy' set is 'fuzzy' in the sense that the boundaries of set membership are not absolute; membership in a fuzzy set can range from 0%–100% (Ref. 33). Thus, paratopes might be viewed as sets comprising prototype and nonprototype amino acids, or as sets of amino acids with membership values at 100% or <100%. Typical contact-mediating residues in the hypervariable regions, which contribute to the energetics of binding to cognate ligand and to the energetics of discrimination between cognate and noncognate ligands, would represent prototype members of the set. Amino acids in the C domains that influence segmental flexibility[21] or inter-Ab association[34,35], and residues in the V domains that are not contact residues but that determine the canonical conformations of hypervariable loops[36] or otherwise influence contact residues, are possible examples of nonprototype members of the paratope set.

Similar logic can be applied to epitopes. Traditionally, a multivalent Ag such as the cell-wall polysaccharide of group A streptococci is viewed, with respect to mAbs specific for the GlcNAc residues of the cell wall polysaccharide, as GlcNAc epitopes with intervening, inert scaffolding. An alternative perspective is that these intervening structures contribute to the specificity of these epitopes, as 'perceived' by an intact IgG mAb. If these structural features, normally excluded from the epitope set, do not contribute as fully to the affinity or specificity of the interaction with an Ab, then the corresponding structural elements could be viewed as nonprototype members of the set or as having <100% membership in the set.

Typically, immunologists have preconceptions regarding the compartmentalization of a molecule into subunits for the purpose of establishing relationships between structural elements and functions. However, there is no scientific or logical basis for presuming that the structural basis for the 'physiology' of a system can be uniquely defined, especially when the structural elements of the system are highly interactive[37]. The suggestion offered above, to consider paratopes and epitopes in broader terms than is traditionally the case, can be viewed as an alternative decomposition of Ab (or Ag) structure into functional subunits. The key point is that the traditional approach to relating Ab (or Ag) structure and specificity is clearly imperfect for the many biologically relevant multivalent interactions.

Concluding remarks

There are practical implications associated with the notion of paratopes as nonclassical sets of amino acids. For example, we would suggest that, in engineering Ab combining sites, optimization of affinity for monovalent and multivalent interactions may not always result from the same amino acid substitutions. Similar speculations would apply to epitopes and Ags. Thus, such effects could be of relevance for the design either of vaccines or of immunotherapeutic soluble receptors for multivalent targets[34].

The arguments presented above should be considered in a broader context. Virtually any important biological concept, when examined in detail, comprises a family of related concepts that overlap to different degrees and in different ways. Evolutionary biology provides numerous examples of central concepts with several interpretations[38]. Multiple meanings co-exist for concepts such as epitope, paratope or specificity, and which meaning is the most appropriate will be dictated, at least in part, by the nature of the investigational objectives.

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Myasthenia gravis and myasthenic syndromes

The Association Française contre les Myopathies (AFM) supports research projects in the fields of neuromuscular diseases and genetic disorders. During the second semester of 1995, the AFM is planning to sponsor a coordinated research effort on myasthenia gravis and related disorders.

Basic, applied and clinical research projects relating to the following themes will be encouraged:

- **Immuno-intervention (induction of self-tolerance, regulation of mechanisms of selection and activation of thymic and peripheral autoreactive lymphocytes, regulation of autoantibody production).**
- **Protection of the motor end plate and facilitation of neuromuscular transmission.**
- **Studies of genetic and other predisposing factors.**

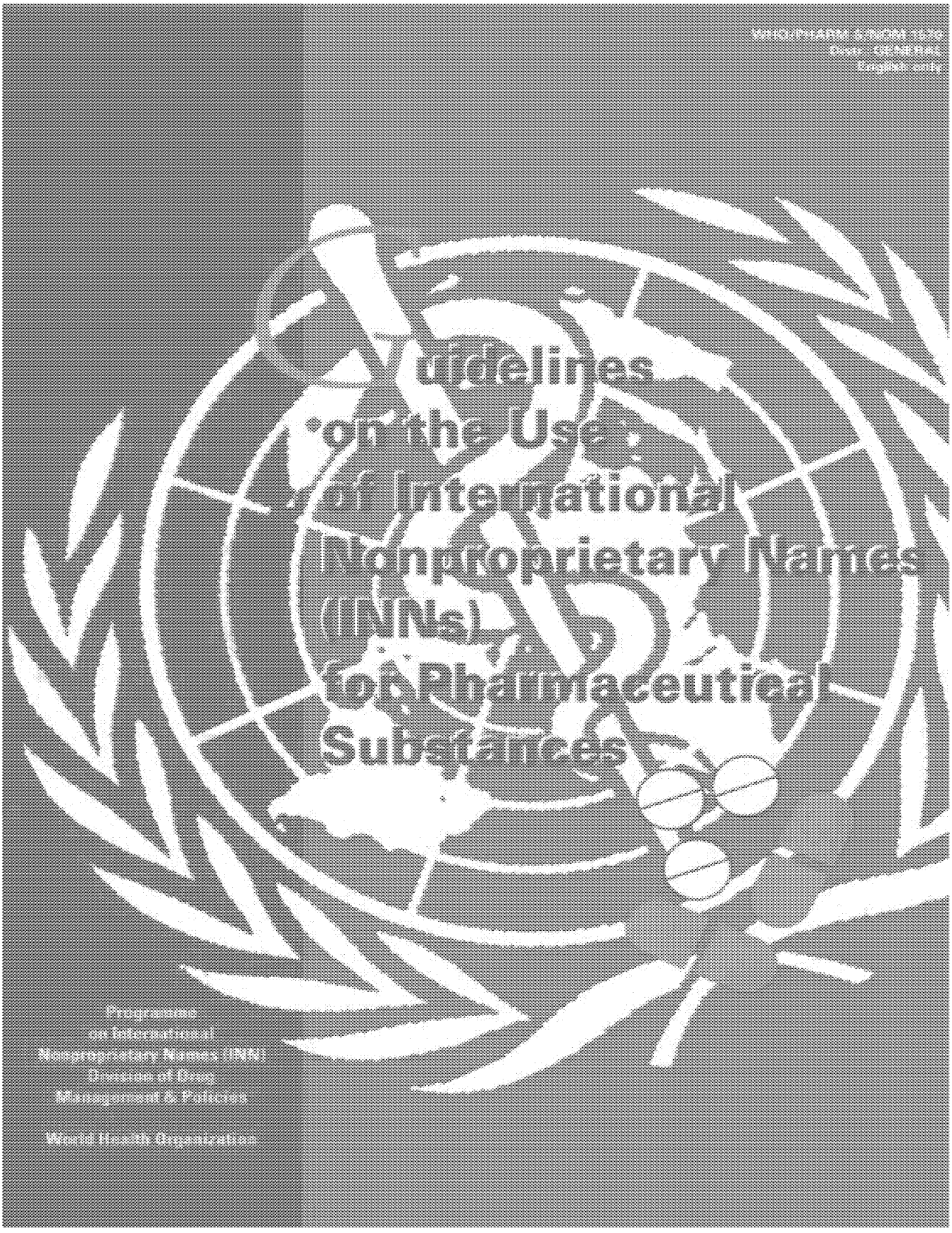
Please send a letter of declaration of intention (two pages maximum) before 30 June 1995 to:

Dominique Duguet,
Secrétariat Permanent du Conseil Scientifique,
Association Française contre les Myopathies,
13 place de Rungis,
75013 Paris, France.
Tel: 33 1 44 16 27 15
Fax: 33 1 45 80 37 36

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Guidelines on the Use of International Nonproprietary Names (INNs) for Pharmaceutical Substances

Programme on International Nonproprietary Names (INN)
Quality Assurance and Safety: Medicines
Essential Medicines and Health Products

World Health Organization

ALXN2046
IPR2019-00740
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WHO/ PHARM S/NOM 1570
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GUIDELINES ON THE USE OF INTERNATIONAL NONPROPRIETARY NAMES (INNs) FOR PHARMACEUTICAL SUBSTANCES



Programme on International Nonproprietary Names (INN)
Division of Drug Management & Policies
World Health Organization
Geneva

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GUIDELINES ON THE USE OF INTERNATIONAL NONPROPRIETARY NAMES (INNs) for Pharmaceutical Substances

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Annex 3 : List of common INN stems
Annex 4 : Specific groups of biological compounds
Annex 5 : WHA resolution on nonproprietary names for pharmaceutical substances (WHA46.19)
Annex 6 : Procedure for the selection of international nonproprietary names for pharmaceutical substances
Annex 7 : Addresses of national nomenclature commissions
Annex 8 : INN request form

Information on the INN Programme and the INN request form
are available on INTERNET:
http://www.who.ch/programmes/dmp/innmain.htm

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1. General introduction

The present guidelines on the use of INNs are intended as a general explanation of the INN selection process. They have been developed for drug regulatory authorities for use in the marketing authorization/ registration of products, drug manufacturers who are requesting new INNs and those using INNs, patent authorities/offices, trade-mark attorneys and trade-mark specialists, scientists, teachers, health professionals, as well as any person interested in nomenclature.

1.1 General information on the INN system

An International Nonproprietary Name (INN) identifies a pharmaceutical substance or active pharmaceutical ingredient by a unique name that is globally recognized and is public property. A nonproprietary name is also known as a generic name.



The INN system as it exists today was initiated in 1950 by a World Health Assembly resolution WHA3.11 and began operating in 1953, when the first list of International Nonproprietary Names for pharmaceutical substances was published. The cumulative list of INNs now stands at some 7000 names designated since that time, and this number is growing every year by some 120 - 150 new INNs.

Since its inception, the aim of the INN system has been to provide health professionals with a unique and universally available designated name to identify each pharmaceutical substance. The existence of an international nomenclature for pharmaceutical substances, in the form of INNs, is important for the clear identification, safe prescription and dispensing of medicines to patients, and for communication and exchange of information among health professionals and scientists worldwide.

As unique names, INNs have to be distinctive in sound and spelling, and should not be liable to confusion with other names in common use. To make INNs universally available they are formally placed by WHO in the public domain, hence their designation as "nonproprietary". They can be used without any restriction whatsoever to identify pharmaceutical substances.

Another important feature of the INN system is that the names of pharmacologically-related substances demonstrate their relationship by using a common "stem". By the use of common stems the medical practitioner, the pharmacist, or anyone dealing with pharmaceutical products can recognize that the substance belongs to a group of substances having similar pharmacological activity. For example all iodine-containing contrast media are given the prefix *io-*, while all ß-adrenoreceptor antagonists the suffix *-olol*. The use of stems is described later in more detail.

The extent of INN utilization is expanding with the increase in the number of names. Its wide application and global recognition are also due to close collaboration in the process of INN selection with numerous national drug nomenclature bodies. The increasing coverage of the drug-name area by INNs has led to the situation whereby the majority of pharmaceutical substances used today in medical practice are designated by an INN. The use of INNs is already common in research and clinical documentation, while

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the importance of the programme is growing further due to expanding use of generic names for pharmaceutical products.

The names which are given the status of an INN are selected by the World Health Organization on the advice of experts from the WHO Expert Advisory Panel on the International Pharmacopoeia and Pharmaceutical Preparations. The process of INN selection follows three main steps:

- a request/application is made by the manufacturer or inventor,
- after a review of the request a proposed INN (prop. INN) is selected and published for comments,
- after a time-period for objections has lapsed, the name will obtain the status of a recommended INN (rec. INN) and is published as such.

The procedures relating to each of these steps are described in the present document in full detail.

INNs are selected in principle only for single, well-defined substances that can be unequivocally characterized by a chemical name (or formula). It is the policy of the INN programme not to select names for mixtures of substances, while substances that are not fully characterized are included in the INN system in exceptional cases only. INNs are not selected for herbal substances (vegetable drugs) or for homoeopathic products. It is also the policy of the INN programme not to select names for those substances that have a long history of use for medical purposes under well-established names such as those of alkaloids (e.g. morphine, codeine), or trivial chemical names (e.g. acetic acid).

The INN is usually designated for the active part of the molecule only, to avoid the multiplication of entries in cases where several salts, esters, etc. are actually used. In such cases, the user of the INN has to create a modified INN (INNM) himself ; *mepyramine maleate* (a salt of *mepyramine* with *maleic acid*) is an example of an INNM. When the creation of an INNM would require the use of a long or inconvenient name for the radical part of the INNM, the INN programme will select a short name for such a radical (for example, *mesilate* for *methanesulfonate*).

Names of pharmaceutical preparations, such as used in pharmacopoieal monograph titles, usually consist of two elements, the first designating the active substance (an INN is used here), and the other designating the dosage form of the product. Rules for creating such names fall outside the INN programme and are not discussed here.

In the process of INN selection, the rights of existing trade-mark owners are fully protected. If in the period of four months following the publication of a proposed INN, a formal objection is filed by an interested person who considers that the proposed INN is in conflict with an existing trade-mark, WHO will actively pursue an arrangement to obtain a withdrawal of such an objection or will reconsider the proposed name. As long as the objection exists, WHO will not published it as a recommended INN.

With the growing number of INNs and trade-marks, the possibility of conflicts between the two has gradually increased, even with full protection of the rights of existing trade-marks. The main source of conflict is usually an attempt by a manufacturer to propose a new trade-mark containing stems established in the INN programme. If protection is granted to such a name, this may diminish the freedom of the INN programme in selecting further INNs in the same series of substances. To prevent such occurrences, the matter was taken up in a resolution of the World Health Assembly *WHA46.19*. This issue is discussed in more detail in section 4.

Further background information on the INN programme may be found in Annex 1.

1.2 Use of INNs

Nonproprietary names are intended for use in pharmacopoeias, labelling, product infromation, advertising and other promotional material, drug regulation and scientific literature, and as a basis for product names, e.g. for generics. Their use is normally required by national or, as in the case of the European Community, by international legislation. As a restult of ongoing collaboration, national names such as British Approved Names (BAN), Dénominations Communes Françaises (DCF), Japanese Adopted Names (JAN) and United States Accepted Names (USAN) are nowadays, with rare exceptions, identical to the INNs.

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Some countries have defined the minimum size of characters in which the generic nonproprietary name must be printed under the trade-mark labelling and advertising. In several countries the generic name must appear prominently in type at least half the size of that used for the proprietary or brand-name. In some countries it has to appear larger than the trade-mark name. Certain countries have even gone so far as to abolish trade-marks within the public sector.

To avoid confusion, which could jeopardize the safety of patients, trade-marks cannot be derived from INNs and, in particular, must include their common stems. As already menitoned the selection of further names within a series will be seriously hindered by the use of a common stem in a brand-name.

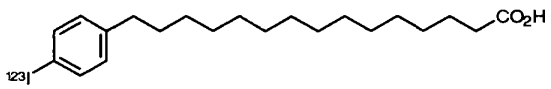
2. Elements in the INN system

2.1 Proposed INNs

The selection of a new INN relies on a a strict procedure. Upon receipt of an INN request form, the WHO Secretariat examines the suggested names for conformity with the general rules, for similarities with published INNs and potential conflicts with existing names, including published INNs and trademarks. A note summarizing the result of these checks is added and the request is subsequently forwarded to the INN experts for comments. Once all experts agree upon one name, the applicant is informed of the selected name.

Newly selected, proposed INNs are then published in *WHO Drug Information*, which indicates a deadline for a 4-month objection period. This period is allowed for comments and/or objections to the published names to be raised. The reasons for any objection must be stated clearly and these will be evaluated by the experts for further action. Users are invited to refrain from using the proposed name until it becomes a recommended INN, in order to avoid confusion should the name be modified.
Two lists of proposed INNs are published yearly. An example is set out below.

acidum iocanlidicum (^{123}I)	
iocanlidic (^{123}I) acid	15-(*p*-[^{123}I]iodophenyl)pentadecanoic acid *radiodiagnostic agent*
acide iocanlidique (^{123}I)	acide 15-(4-[^{123}I]iodophényl)pentadécanoïque *produit à usage radiodiagnostique*
ácido iocanlídico (^{123}I)	ácido 15-(*p*-[^{123}I]iodofenil)pentadecanoico *agente de radiodiagnóstico*
	$C_{21}H_{33}{}^{123}IO_2$ 74855-17-7



2.2 Recommended INNs

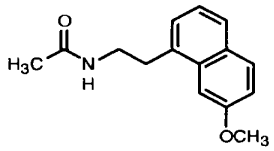
The final stage of the selection process is the recommended INN. Once a name has been published as a recommended INN it will not normally be modified further and is ready for use in labelling, publications, on drug information. It will serve to identify the active pharmaceutical substance during its life-time worldwide. Since the name is available in the public domain it may be used freely. However, it should not be registered as a trademark since this would prevent its use by other parties (see also chapter 4.).

Recommended INNs are published in the *WHO Drug Information* as a consequence of the objection procedure applied to proposed INNs (see 2.1 above). As from 1997, two lists of proposed INNs are published yearly and as from list 37 of recommended INNs, graphic formulae are also included for better identification of the substances.

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An example of an entry in the list may be found below:

agomelatinum	
agomelatine	*N*-[2-(7-methoxy-1-naphthyl)ethyl]acetamide
agomélatine	*N*-[2-(7-méthoxynaphtalén-1-yl)éthyl]acétamide
agomelatina	*N*-[2-(7-metoxi-1-naftil)etil]acetamida
	$C_{15}H_{17}NO_2$



2.3 Names for radicals and groups

During the 1975 meeting on Nonproprietary Names for Pharmaceutical Substances the experts discussed the issue of INNs for salts and esters and noted that requests had frequently been received for INNs for salts, esters, or combination products of substances for which INNs already existed. At that time, the experts decided that INNs for the simple salt and esters should be devised from the INN in conformity with normal chemical practice.

Some of the radicals and groups involved are, however, of such complex composition that it makes it inconvenient to use the chemical nomenclature. It was thus decided that in such cases, shorter nonproprietary names are selected for these inactive moieties and published in proposed lists under the title "Names for Radicals and Groups". Separate names for salts and esters derived from this procedure are not published. If a "radical and group name" is used in conjunction with an INN, they are referred to as International Nonproprietary Name (Modified) or INNM.

A comprehensive list of radicals and groups may be obtained from the Distribution and Sales unit or the INN Secretariat (*INNs: Names for radicals and groups, combined summary list, WHO/PHARM S/NOM 1506, updated regularly*).

2.4 Modified INNs (INNMs)

In principle, INNs are selected only for the active part of the molecule which is usually the base, acid or alcohol. In some cases, however, the active molecules need to be expanded for various reasons, such as formulation purposes, bioavailability or absorption rate. In 1975 the experts designated for the selection of INN decided to adopt a new policy for naming such molecules. In future, names for different salts or esters of the same active substance should differ only with regard to the inactive moiety of the molecule. For example, *oxacillin* and *ibufenac* are INNs and their salts are named *oxacillin sodium* and *ibufenac sodium*. The latter are called modified INNs (INNMs).

Before the existence of this rule, some INNs were published for salts. In such cases, the term "modified INN" may also be used for a base or acid. For example, *levothyroxine sodium* was published as an INN and *levothyroxine* may thus be referred to as an INNM.

Please see also chapter 2.4 for radicals and groups (see also 2.4) which are used in conjunction with INNs and which are also referred to as INNM.

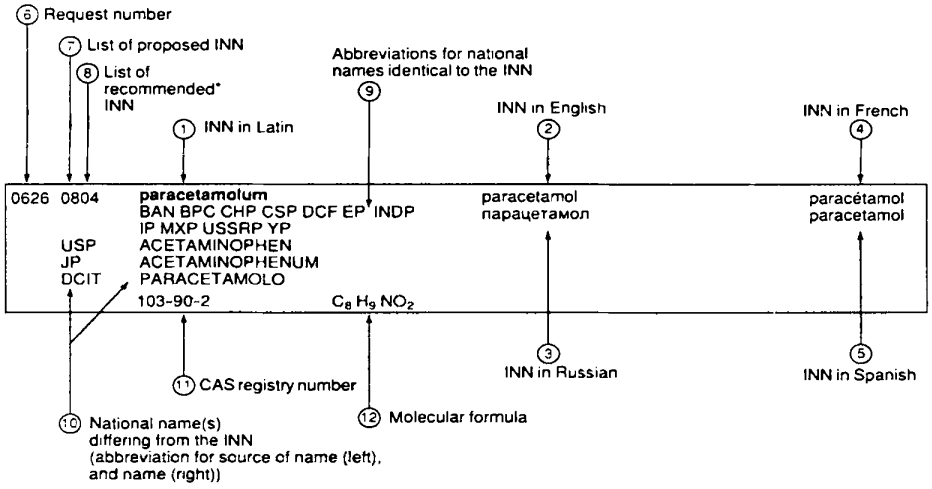
2.5 Cumulative list

All names selected as proposed and recommended INNs are published in a *Cumulative list*, which is updated periodically. The generic names are presented in alphabetical order by Latin name. Each entry includes:

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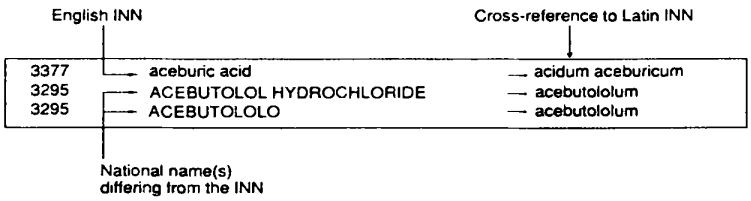
- equivalent nonproprietary names: in Latin, English, French, Russian and Spanish;
- a reference to the INN list in which the name was originally proposed or recommended, or last amended;
- reference to names of substances that have been abandoned or never been marketed;
- reference to national nonproprietary names;
- reference to pharmacopoeial monographs or similar official references;
- reference to names issued by the International Organization for Standardization (ISO);
- reference to the Convention of Psychotropic Substances, if applicable;
- reference to the List of Narcotic Drugs under International Control, if applicable;
- the molecular formula;
- its Chemical Abstracts Service (CAS) number.

The layout for information contained in the *Cumulative list* of INNs is as follows:



* An asterisk in place of a recommended list number signifies that an objection has been raised to the proposed name.

Note: Cross-references are provided for entries corresponding to (a) English, French and Spanish INN that appear in different alphabetical positions from the Latin INN and (b) national names that differ from the INN. Entries for (a) are printed in lower-case letters (as in the example of aceburic acid, below) while entries for (b) are printed in capitals (as in the examples of ACEBUTOLOL HYDROCHLORIDE and ACEBUTOLOLO).



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3. Principles for selection of INNs

3.1 General rules

General rules were established at the beginning of the INN programme in order to guide the members of the INN committee and to allow health professionals to understand the rationale for a number of new names. At first, some countries used shortened chemical names as generic names, but this system was found to be very limited, since many molecules contain similar elements and groups, such as phenol, chlor, methyl or benzene-rings, in their chemical structures. In addition, a name that indicates relationship to a group of pharmacological similarly-acting substances is more meaningful to users.

In its Twentieth Report (WHO Technical Report Series, No. 581, 1975), the WHO Expert Committee on Nonproprietary Names for Pharmaceutical Substances reviewed the general principles for devising, and the procedures for selecting, international nonproprietary names (INN) in the light of developments in pharmaceutical compounds over the years. The current version of the *General principles for guidance in devising international nonproprietary names for pharmaceutical substances* is reproduced in Annex 2.

3.2 Use of stems

Usually, an INN consists of a random, fantasy prefix and a common stem; substances belonging to a group of pharmacologically related substances show their relationship by the use of a common stem. Sometimes sub-stems are established to differentiate between different related groups of substances, e.g. *-olol* for b-adrenoreceptor antagonists and antihypertensives, *-teplase* for tissue-type-plasminogen activators and *-uplase* for urokinase-type-plasminogen activators.

A list of common stems used in the selection of INNs may be found in Annex 3.

3.3 Stereoisomers

An INN for a new chemical entity does not routinely specify the stereoisomeric state of the molecule in the nonproprietary name. If the stereochemistry has been determined, then this information is presented in the chemical name(s) to identify the substance. An INN can, therefore, identify the racemic mixture (e.g. ibuprofen, tetramisole), the *levo-* isomer (e.g. amifostine, lofentanil, prenalterol, remoxipride, quadazocine), or the *dextro* form (e.g. butopamine). Subsequently if an INN is needed for a different enantiomer or for the racemic form, the following prefixes should be added to the existing INN:

(a) For the *levo* form, the *lev-/levo-* prefix is used, e.g. levocarnitine, levamisole.
(b) For the *dextro* form, the *dex-* prefix is used, e.g. dexamisole, dexibuprofen.
(c) For the *racemic* form, the *rac-/race-* prefix is used, e.g. racepinefrine.

3.4 Radioactive compounds

A name for a drug substance containing a radioactive atom should list, in the following order:

(1) the name of the substance containing the radioactive atom,
(2) the isotope number,
(3) the element symbol, and
(4) the name of the carrier agent, if any,

e.g. cyanocobalamin (^{60}Co), technetium (^{99m}Tc) bicisate, technetium (^{99m}Tc) sestamibi.

3.5 Specific groups of biological compounds

Because of the complexity of certain new types of pharmaceutical products, such as compounds produced by biotechnology, general rules are not always easily formulated. Some of these substances may already have descriptive names assigned by other institutions such as the *International Union of Biochemistry (IUB), International Union of Pure and Applied Chemistry* (IUPAC), *Joint Commission on Biochemical Nomenclature* (JCBN). These names may not be suitable as INNs.

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Annex 4 summarizes nomenclature schemes for groups of biological compounds (for details and examples please also see document WHO/PHARM S/NOM 15: *The use of common stems in the selection of international nonproprietary names (INN) for pharmaceutical substances,* updated regularly*).*

4. Protection of INNs

Lists of both proposed and recommended INNs are sent together with a *note verbale* by the Director-General to WHO Member States (at present 191), to national pharmacopoeia commissions and to other bodies designated by Member States. In his *note verbale,* the Director-General of the World Health Organization requests that Member States should take such steps as are necessary to prevent the acquisition of proprietary rights on the name, including prohibiting registration of the name as a tradename.

Over the years, the need to maintain the integrity of the INN system has become urgent. This is reflected in the following extract from the Fifth Report of the WHO Expert Committee on the Use of Essential Drugs which met in November 1991[1]:

> **"The procedure for selecting INNs allows manufacturers to contest names that are either identical or similar to their licensed trade marks. In contrast, trade mark applications are disallowed, in accordance with the present procedure, only when they are identical to an INN.A case for increased protection of INNs is now apparent as a result of competitive promotion of products no longer protected by patents. Rather than marketing these products under generic name, many companies apply for a trade mark derived from an INN and, in particular, including the INN common stem. This practice endangers the principle that INNs are public property; it can frustrate the rational selection of further INNs for related substances, and it will ultimately compromise the safety of patients by promoting confusion in drug nomenclature."**

These concerns were debated during the sixth International Conference of Drug Regulatory Authorities (ICDRA), in Ottawa, in October 1991.

Based on recommendations made by the WHO Expert Committee on the use of Essential Drugs the resolution WHA46.19 on *Nonproprietary Names for pharmaceutical substances* was adopted in May, 1993 during the Forty-sixth World Health Assembly requesting Member States to:

> ■ ***"enact rules or regulations, as necessary, to ensure that international nonproprietary names (or the equivalent nationally approved generic nam used in the labelling and advertising of pharmaceutical products are always displayed prominently;***
> ■ ***to encourage manufacturers to rely on their corporate name and the international nonproprietary names, rather than on trade-marks, to promote and market multisource products introduced after patent expiration;***
> ■ ***to develop policy guidelines on the use and protection of international nonproprietary names,and to discourage the use of names derived from INNs, and particularly names including established INN stems as trade-marks."***

In the Director General's *note verbale* attention is drawn to this resolution concerning the use and protection of International Nonproprietary Names (INNs).
The full text of the resolution is reproduced in Annex 5.

As a matter of principle, it may thus be recommended that trade marks should not be derived from INNs. In particular, the intentional incorporation of meaningful INN stems in trade marks should be avoided.

Similarly, inclusion of elements from biochemical nomenclature (like *-feron* from interferon, or *-leukin* from interleukin) in trade marks in anticipation is discouraged since these elements are likely to be utilized as stems within the INN nomenclature. Their inclusion in trade marks could pre-empt the logical development of the INN nomenclature.

[1] WHO Technical Report Series, No. 825, 1992.

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In accordance with resolution WHA46.19, registration of an INN together with a firm's name is perfectly acceptable, as long as it does not prevent another manufacturer from using the same approach.

5. How to apply for an INN

5.1 Procedure for selection of INNs

The selection of INNs is based on the *Procedure for selection of international nonproprietary names for pharmaceutical substances.* The text adopted is set out in World Health Assembly resolution WHA3.11 [Text adopted by the Executive Board of WHO in resolution EB15.R7 (*Off. Rec. Wld Health Org.*, 1955, **60**, 3) and amended by the Board in resolution EB43.R9 (*Off. Rec. Wld Hlth Org.*, 1969, **173**, 10).]. The application/request form for INN is attached as Annex 6 in its updated version.

In countries with national nomenclature commissions, applications for international nonproprietary names should be made through the national authorities (addresses - see Annex 7). In countries without a national nomenclature commission, requests for INNs may be forwarded directly to WHO. Applications for INNs should be addressed to:

Secretary of the INN Programme
Quality Assurance - INNs
Drug Management and Policies
World Health Organization
20, Avenue Appia
CH-1211 Geneva 27
***Internet:* koppkubels@who.ch**
***Fax* : +41 22 791 07 46**
***Tel.* : +41 22 791 36 36/36 60**

5.2 INN request form

Before a suggested name can be evaluated by the INN Secretariat, complete information must be provided on a request form to facilitate uniform handling of the data and to assure that pertinent items have not been omitted. It is important that the information is as comprehensive as possible. If parts of this information are missing or explanations are unclear or incomplete, the INN Secretariat will request the applicant to furnish the missing data. This can result in delay because selection of an INN requires the availability of all relevant information to the INN experts.

The following explanations will help applicants to complete the INN form. If additional information is needed, an applicant may contact the INN Secretariat at the World Health Organization, DMP/QAS, 20 Avenue Appia, CH-1211 Geneva 27, Switzerland. (Telephone: +41/22/791 36.36/36.60. Facsimile: +41/22/ 791.07.46. Internet: *koppkubels@who.ch*).

Suggested names in order of preference

An applicant may make 3 suggestions for an INN relating to the acid, base or alcohol of a specific chemical entity under investigation. The suggested name should be a single word and not inconveniently long.

Nonproprietary names are developed by a system that relates compounds with chemical, pharmacological or therapeutic similarity. Therefore, whenever justified, the suggested name must incorporate the established common stem. A list of stems may be found in the document entitled *The use of common stems in the selection of international nonproprietary names (INN) for pharmaceutical substances (WHO/PHARM S/NOM 15)* which is updated regularly.

Occasionally stems require modification. For example, some drugs inhibit a-adreno-receptors as well as ß-adrenoreceptors and exhibit a specific structural variation from the "*-olol*" prototype. Accordingly, for this type of drug, the stem was modified by one letter to "*-alol*". This change introduces a nuance in the

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naming of related groups of drugs that may not be apparent to every observer but would be understood by someone familiar with the naming conventions of the ß-adrenoreceptor antagonists and related compounds. The important point is that similar compounds have a common element in the name that imparts useful information.

It is imperative that the newly suggested name does not conflict with existing chemical names, other nonproprietary names or trade-marks. Therefore, the INN Secretariat requests the applicant to verify the absence of conflicts with existing chemical names, common names for insecticides, other nonproprietary names, and trade-marks. Some firms routinely perform exhaustive searches for possible conflicts with a suggested INN and for pharmacologically and chemically related compounds with already assigned INNs; the INN Secretariat would appreciate receiving this information to avoid search duplication.

Chemical name and description

Chemical information should be as complete and as current as possible. Information on stereochemistry should be included if known. The chemical names will be in accordance with the nomenclature rules of the **I**nternational **U**nion of **P**ure and **A**pplied **C**hemistry (**IUPAC**) as interpreted by the Chemical Abstracts Service (8th collective period); the Chemical Abstracts Index names in their current style may also be included as additional information. The chemical name provided by the manufacturer is reviewed for accuracy and to confirm that its construction follows accepted chemical nomenclature rules.

A description is used to identify a substance that is insufficiently defined to be assigned an IUPAC and CAS chemical name. This description will be superseded by the chemical name when the drug substance is fully characterized.

Precautions are taken to ensure confidentiality of the material submitted to WHO, but an applicant should not attempt to obtain an INN before all patent procedures are completed and until full chemical information can be made available to WHO.

Graphic formula

Without a graphic formula, it may be difficult to determine if an INN already exists. In addition, the graphic formula is necessary to relate the new drug to existing compounds in the same chemical family. Guidelines for drawing structures may be found in the document entitled *Graphic representation of chemical formulae in the publications of International Nonproprietary Names for pharmaceutical substances (WHO/PHARM/95.579)*, available from the INN Secretariat upon request.

Molecular formula

A one-line molecular formula constructed in accordance with accepted chemical practices should be supplied. The molecular formulas should be given in the following manner, e.g. $C_{21}H_{28}N_2$.

Chemical Abstracts Service (CAS) registry number

If a CAS registry number has been assigned to a new compound before it is submitted to the INN Secretariat, the number should be included on the form. If no number has yet been assigned, the manufacturer should obtain the CAS registry number from Chemical Abstracts Services for publication in the INN lists. Proof of the entry will be required.

Tradenames (known or contemplated)

If a trade-mark has been issued for the drug, it should be entered on the form. List any national or international trade-marks (and manufacturers) and the name of the country where the trade-mark is registered.

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Any other name or code

Sometimes, long before a nonproprietary name or a trade-mark has been selected for a new compound, it may acquire a trivial name that has been used in the laboratory and scientific literature. The INN Secretariat would like to be made aware of such names but requests manufacturers not to create, use, or in any way encourage the creation of trivial names for new drugs. The fact that a trivial name has become accepted in the literature will not ensure its adoption as a nonproprietary name and may only cause confusion when an official nonproprietary name is selected. It is therefore recommended to use codes before the publication of a recommended nonproprietary name and indicate these on the request form to the INN Secretariat as an additional reference.

Principal therapeutic use(s) and posology

It is important to know the therapeutic category for the new compound as such information may determine the stem selected for the nonproprietary name. Pertinent reprints presenting evidence of the claimed therapeutic use should be included with the application (for terminology, please see *Pharmacological Action and Therapeutic Use of Drugs, a list of terms*, English/French/Spanish, 1996 (*PHARM/96.320*).

Pharmacological action

The pharmacological action should be explained in as much detail as possible, since it may also influence the stem selected for the compound. Again, pertinent reprints must be included to support the claimed action (for terminology, please see above).

Verso side of request form

Date of clinical trial

As a general guide, the development of a drug should progress up to the point of clinical trials (phase II) before an application is submitted to the INN Secretariat for name selection. An approximate date when clinical trials began is requested. The intent of this request is to assure that clinical trials are under way. It is the belief that if a drug has entered clinical trials, there is a reasonable expectation that it will be marketed and thus the name selected will have been developed for that need and purpose.

In case the development is stopped, the manufacturer should inform the INN Secretariat as soon as possible, in order to halt the selection process.

Availability of suggested names

The originator of the INN request confirms with his signature that the suggestion is made on the understanding that, insofar as is known, none of the suggested names are either registered or pending registration.

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Permission to publish the CAS registry number

The applicant herewith confirms that the CAS registry number sent to the INN Secretariat is correct and may be used in the INN lists.

Additional comments

This section allows the applicant to give additional comments and/or information.

6. References for supporting material

Documents:

- *The use of common stems in the selection of international nonproprietary names (INN) for pharmaceutical substances* (WHO/PHARM S/NOM 15) INN Programme, WHO, Geneva - *updated regularly*
- *Graphic representation of chemical formulae in the publications of international nonproprietary names (INN) for pharmaceutical substances* (WHO/PHARM/95.579), INN Programme, WHO, Geneva
- *Pharmacological Action and Therapeutic Use of Drugs, list of terms*, English/French Spanish, 1996, (*PHARM/96.320),* WHO, Geneva
- *INNs: Names for radicals and groups, combined summary list,* WHO/PHARM S/NOM1506, INN Programme, WHO, Geneva - *updated regularly*
- *Definition of INNs for Substances Prepared by Biotechnology,* PHARM S/NOM 1348,INN Programme, WHO, Geneva

Publications:

- *Cumulative List of INNs, No. 9,* 1996, WHO, Geneva
- *WHO Drug Information* (quarterly journal published by the World Health Organization)

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ANNEX 1

Background information on the INN Programme

The activities of national nomenclature commissions are coordinated in order to achieve international standardization in nomenclature under the auspices of WHO according to *article 2a* and *2u* of its constitution (adopted in 1946 in New York):

"In order to achieve its objective, the functions of the World Health Organization shall be:

(a) to act as the directing and coordinating authority on international health work; ...
(u) to develop, establish and promote international standards with respect to food, biological, pharmaceutical and similar products; ..."

The WHO programme on the selection of international nonproprietary names (INN) emerged really as an extension of the WHO programme on the unification of pharmacopoeias and the preparation of the International Pharmacopoeia requested by the very first World Health Assembly in July 1948 in resolution *WHA1.27*. The meeting of an Expert Committee on Unification of Pharmacopoeias in 1949 studied the preparation of general rules for nomenclature, and drew up a plan that was adopted in 1950 by a resolution of the World Health Assembly (*WHA3.11*).

The World Health Organization's (WHO) international nomenclature programme was thus established in 1953 when Member countries adopted a resolution at the World Health Assembly officially initiating the programme on **I**nternational **N**onproprietary **N**ames (**INN**) for pharmaceutical substances [French : **D**énominations **C**ommunes **I**nternationales]; [Spanish : **D**enominaciones **C**omunes **I**nternacionales - **DCI**].

The official "Procedure for the Selection of Recommended International Nonproprietary Names for Pharmaceutical Substances" and the "General Principles for Guidance in Devising International Nonproprietary Names for Pharmaceutical Substances" on which the whole programme is based were adopted by the Executive Board in 1955 in resolution *EB15.R7*. The Procedure has remained unchanged -- except for the replacement of the words "INNs for Pharmaceutical Preparations" by "*INNs for Pharmaceutical Substances*" (res. *EB43.R9*). However, the General Principles have evolved and revisions were regularly approved in the reports of the Sub-Committee meetings submitted to the Executive Board. Since 1969 the Director-General is authorized by the Executive Board to make such revisions of the General Principles as may seem desirable in the light of advances in science and of experience as may be suggested by the members of the Expert Advisory Panel on the International Pharmacopoeia and Pharmaceutical Preparations designated to deal with the selection of nonproprietary names (INN experts) in accordance with the above-mentioned Procedure (res. *EB37.R9*).

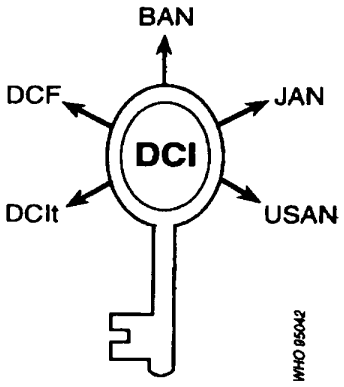
From 1950 onwards the programme was dealt with by the Sub-Committee of the Expert Committee on the Unification of Pharmacopoeias. The first task of the Sub-Committee was to establish contacts with national pharmacopoeia commissions that had already established programmes on the unification of drug nomenclature as those carried out under the *Comité de Nomenclature* of the *Commission permanente de la Pharmacopée Française*, the Nomenclature Committee of the British Pharmacopoeia Commission, the Council of Drugs of the American Medical Association in the USA and the Nomenclature Committee of the Nordic Pharmacopoeia Council in the Scandinavian countries. The purpose of these contacts was to coordinate the activities of such existing national nomenclature programmes.

Between 1950 and 1966 the Sub-Committee met 16 times. The earlier meetings were concerned with developing the Procedure and the General Principles and the first list of proposed INNs was only published in 1953. In 1967 the Sub-Committee became the Expert Committee on Nonproprietary Names for Pharmaceutical Preparations and later the Expert Committee on Nonproprietary Names for Pharmaceutical Substances. This Expert Committee only met in 1967, 1968, 1970 and 1975. In the other years, and since 1976, the meetings were held in a less formal way and

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referred to as Consultations on the Selection of INNs. The justification for this less formal approach is that the main report of the Committee consists of the officially published INNs selected during its meetings.

The composition of the INN meetings over almost 40 years has been characterized by a great stability. The average number of participants is 6-8 experts, mostly people with responsible positions in - or secretaries of - national nomenclature commissions, and only some 30 people have been involved over the years. At present the Expert group is composed of experts from France, Indonesia, Japan, Nigeria, Poland, Spain, the United Kingdom and USA.



BAN: British Approved Name
DCF: Dénomination Commune Française
DCIt: Denominazione Comune Italiana
JAN: Japanese Accepted Name
USAN: United States Approved Name

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ANNEX 2

General principles for guidance in devising International nonproprietary names for pharmaceutical substances

1. International Nonproprietary Names (INN) should be distinctive in sound and spelling. They should not be inconveniently long and should not be liable to confusion with names in common use.

2. The INN for a substance belonging to a group of pharmacologically related substances should, where appropriate, show this relationship. Names that are likely to convey to a patient an anatomical, physiological, pathological or therapeutic suggestion should be avoided.

These primary principles are to be implemented by using the following secondary principles:

3. In devising the INN of the first substance in a new pharmacological group, consideration should be given to the possibility of devising suitable INN for related substances, belonging to the new group.

4. In devising INN for acids, one-word names are preferred; their salts should be named without modifying the acid name, e.g. "oxacillin" and "oxacillin sodium", "ibufenac" and "ibufenac sodium".

5. INN for substances which are used as salts should in general apply to the active base or the active acid. Names for different salts or esters of the same active substance should differ only in respect of the name of the inactive acid or the inactive base.

For quaternary ammonium substances, the cation and anion should be named appropriately as separate components of a quaternary substance and not in the amine-salt style.

6. The use of an isolated letter or number should be avoided; hyphenated construction is also undesirable.

7. To facilitate the translation and pronunciation of INN, "f" should be used instead of "ph", "t" instead of "th", "e" instead of "ae" or "oe", and "i" instead of "y"; the use of the letters "h" and "k" should be avoided."

When devising an INN it is important to be aware of possible language problems . Since the name is used worldwide, not only should certain letters be avoided, but experts need to be aware of unsuitable connotations in the major languages spoken in the world.

8. Provided that the names suggested are in accordance with these principles, names proposed by the person discovering or first developing and marketing a pharmaceutical preparation, or names already officially in use in any country, should receive preferential consideration.

9. Group relationship in INN (see Guiding Principle 2) should if possible be shown by using a common stem. The following list contains examples of stems for groups of substances, particularly for new groups. There are many other stems in active use. [1]Where a stem is shown without any hyphens it may be used anywhere in the name.

Latin	***English***	
-acum	-ac	anti-inflammatory agents of the ibufenac group
-actidum	-actide	synthetic polypeptides with a corticotropin-like action
-adolum	-adol }	analgesics
-adol-	-adol-}	analgesics
-astum	-ast	antiasthmatic, antiallergic substances not acting primarily as antihistaminics
-astinum	-astine	antihistaminics
-azepamum	-azepam	diazepam derivatives
-bactamum	-bactam	b-lactamase inhibitors

[1] An extensive listing of stems is contained in the working document WHO/PHARM S/NOM15 which is regularly updated and can be requested from the INN Secretariat, WHO, Geneva.

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Latin	*English*	
bol	bol	steroids, anabolic
-buzonum	-buzone	anti-inflammatory analgesics, phenylbutazone derivatives
-cain-	-cain-	antifibrillant substances with local anaesthetic activity
-cainum	-caine	local anaesthetics
cef-	cef-	antibiotics, cefalosporanic acid derivatives
-cillinum	-cillin	antibiotics, derivatives of 6-amino- penicillanic acid
-conazolum	-conazole	systemic antifungal agents, miconazole derivatives
cort	cort	corticosteroids, except prednisolone derivatives
-dipinum	-dipine	calcium channel blockers, nifedipine derivatives
fibratum	-fibrate	clofibrate derivatives
gest	gest	steroids, progestogens
gli-	gli-	sulfonamide hypoglycaemics
io-	io-	iodine-containing contrast media
-ium	-ium	quaternary ammonium compounds
-metacinum	-metacin	anti-inflammatory substances, indometacin derivatives
-mycinum	-mycin	antibiotics, produced by *Streptomyces strains*
-nidazolum	-nidazole	antiprotozoal substances, metronidazole derivatives
-ololum	-olol	b-adrenoreceptor antagonists
-oxacinum	-oxacin	antibacterial agents, nalidixic acid derivatives
-pridum	-pride	sulpiride derivatives
-pril(at)um	-pril(at)	angiotensin-converting enzyme inhibitors
-profenum	-profen	anti-inflammatory substances, ibuprofen derivatives
prost	prost	prostaglandins
-relinum	-relin	hypophyseal hormone release-stimulating peptides
-terolum	-terol	bronchodilators, phenethylamine derivatives
-tidinum	-tidine	histamine H_2-receptor antagonists
-trexatum	-trexate	folic acid antagonists
-verinum	-verine	spasmolytics with a papaverine-like action
vin-	vin-)	vinca alkaloids
-vin-	-vin-)	vinca alkaloids

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ANNEX 3

LIST OF COMMON STEMS USED IN THE SELECTION OF INNs

STEM	*DEFINITION* *substem, if available*
-ac	anti-inflammatory agents, ibufenac derivatives
-actide	synthetic polypeptides with a corticotropin-like action
-adol or -adol-	analgesics
-adom	analgesics, tifluadom derivatives
-afenone	antiarrhythmics, propafenone derivatives
-aj-	antiarrhythmics, ajmaline derivatives
-aldrate	antacids, aluminium salts
-alox	antacids, aluminium derivatives
-amivir see *vir*	
andr or -stan- or -ster-	steroids, androgens
-anserin	serotonin receptor antagonists (mostly 5-HT_2)
-antel	anthelminthics (undefined group)
-apine	see *-pine*
-arabine	arabinofuranosyl derivatives
-arit	antiarthritic substances, acting like clobuzarit and lobenzarit (mechanism different from anti-inflammatory type substances, e.g. *-fenamates* or *-profens*)
-arol	anticoagulants, dicoumarol derivatives
arte-	antimalarial agents, artemisinin related compounds
-ase	enzymes; *-dismase* (superoxide dismutase activity), *-pase* (lipase) *-teplase* (tissue plasminogen activators), *-uplase* (urokinase-type-plasminogen activators)
-ast	antiasthmatic, antiallergics, not acting primarily as antihistaminics; *-lukast (* leukotriene receptor antagonist); *-trodast* (thromboxane A_2 receptor antagonists, antiasthmatics)
-astine	antihistaminics
-azenil	benzodiazepine receptor antagonists/agonists (benzodiazepine derivatives)
-azepam	diazepam derivatives
-azepide	cholecystokinin receptor antagonist
-azocine	narcotic antagonists/agonsists related to 6,7-benzomorphan
-azoline	antihistaminics or local vasoconstrictors, antazoline derivatives
-azosin	antihypertensive substances, prazosin derivatives
-bactam	b-lactamase inhibitors
-bamate	tranquillizers, propanediol and pentanediol derivatives
barb	hypnotics, barbituric acid derivatives
-bendazole	anthelminthics, tiabendazole derivatives
bol	anabolic steroids
-bradine	bradycardic agents

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STEM	***DEFINITION*** ***substem, if available***
-buzone	anti-inflammatory analgesics, phenylbutazone derivatives
-cain-	Class I antiarrhythmics, procainamide and lidocaine derivatives (antifibrillants with local anaesthetic activity)
-caine	local anaesthetics
calci	Vitamin D analogues/derivatives
-carbef	antibiotics, carbacepham derivatives
-carnil	benzodiazepine receptor antagonists/agonists (carboline derivatives)
-cavir	see *vir*
cef-	antibiotics, cefalosporanic acid derivatives
cell- or cel- or -cell-	cellulose derivatives *cell-ate* (cellulose ester derivatives); *-cellose* (cellulose ether derivatives)
-cic	hepatoprotective substances with a carboxylic acid group
-cidin	naturally occurring antibiotics (undefined group)
-cillin	antibiotics, 6-aminopenicillanic acid derivatives
-citabine	nucleoside antiviral or antineoplastic agents, cytarabine or azarabine derivatives
-clone	hypnotic tranquillizers
-cog	blood coagulation factors; *(-)eptacog* (blood coagulation VII); *(-)octocog* (blood coagulation factor VIII); *(-)nonacog* (blood coagulation factor IX)
-conazole	systemic antifungal agents, miconazole derivatives
cort	corticosteroids, except prednisolone derivatives
-crinat	diuretics, etacrynic acid derivatives
-crine	acetylcholinesterase inhibitors, tacrine derivatives
-cromil	antiallergics, cromoglicic acid derivatives
-curium	see *-ium*
-cycline	antibiotics, tetracycline derivatives
-dan	cardiac stimulants, pimobendan derivatives
-dapsone	antimycobacterials, diaminodiphenylsulfone derivatives
-dermin	see *-ermin*
-dil	vasodilators
-dipine	calcium channel blockers, nifedipine derivatives
-dismase	see *-ase*
-dopa	dopamine receptor agonists, dopamine derivatives, used as antiparkinsonism/prolactin inhibitors;
-dox	antibacterials, quinoline dioxide derivatives
-dralazine	antihypertensives, hydrazinephthalazine derivatives
-drine	sympathomimetics; *-frine*: sympathomimetic, phenethyl derivatives
-dronic acid	calcium metabolism regulator, pharmaceutical aid
-ectin	antiparasitics, ivermectin derivatives
-entan	endothelin receptor antagonists
-eptacog	see *-cog*

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STEM	***DEFINITION*** ***substem, if available***
erg	ergot alkaloid derivatives
-eridine	analgesics, pethidine derivatives
-ermin	growth factors; *-dermin* (epidermal growth factors); *-fermin* (fibrinoblast growth factors); *-nermin* (tumour necrosis factor); *-sermin* (insulin-like growth factors)
estr	estrogens
-etanide	diuretics, piretanide derivatives
-exakin	see *-kin*
-exine	mucolytic, bromhexine derivatives
-fenamic acid	anti-inflammatory, anthranilic acid derivatives *-fenamate* ("*-fenamic acid*" derivatives)
-fenin	diagnostic aids; (phenylcarbamoyl)methyl iminodiacetic acid derivatives
-fenine	analgesics, glafenine derivatives - (subgroup of fenamic acid group)
-fentanil	narcotic analgesics, fentanil derivatives
-fermin	see *-ermin*
-fiban	fibrinogen receptor antagonists (glycoprotein IIb/IIIa receptor antagonists)
-fibrate	clofibrate derivatives
-flapon	5-lipoxygenase-activating protein (FLAP) inhibitor
-flurane	general inhalation anaesthetics, halogenated alkane derivatives
-formin	antihyperglycaemics, phenformin derivatives
-fos (-vos)	insecticides, anthelmintics, pesticides etc., phosphorous derivatives
-fos- or fos-	various pharmacological categories belonging to -fos (other than above)
-fradil	calcium channel blockers acting as vasodilators
-frine	see *-drine*
-fungin a	ntifungal antibiotics
-fylline	*N*-methylated xanthine derivatives
gab	gabamimetic agents
gado-	diagnostic agents, gadolinium derivatives
-gatran	thrombin inhibitor, antithrombotic agents
gest	steroids, progestogens
-giline	MAO-inhibitors type B
-gillin	antibiotics produced by *Aspergillus* strains
gli	antihyperglycaemics, sulfonamide derivatives
-golide	dopamine receptor agonists, ergoline derivatives
-gramostim	see *-stim*
-grastim	see *-stim*
-grel- or -grel	platelet aggregation inhibitors
guan-	antihypertensives, guanidine derivatives
-icam	anti-inflammatory, isoxicam derivatives
-ifene	antiestrogens, *clomifene* and *tamoxifen* derivatives
-ilide	Class III antiarrhythmics, sematilide derivatives
imex	immunostimulants

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STEM	***DEFINITION*** ***substem, if available***
-imod	immunomodulators, both stimulant/suppressive and stimulant
-imus	immunosuppressants (other than antineoplastics)
io-	iodine-containing contrast media
-io- or iod-	iodine containing compounds other than contrast media
-iptan	serotonin ($5HT_1$) receptor agonists, sumatriptan derivatives
-irudin	hirudin derivatives
-isomide	antiarrhythmics, disopyramide derivatives
-ium	quarternary ammonium compounds; *-curium* (curare-like substances)
-izine	diphenylmethyl piperazine derivatives; *-rizine* (antihistaminics/cerebral (or peripheral) vasodilators)
-kacin	antibiotics, kanamycin and bekanamycin derivatives (obtained from *Streptomyces kanamyceticus*);
-kalant	potassium channel blockers
-kalim	potassium channel activators, antihypertensive
-kef-	enkephalin agonists
-kin	interleukin type substances; *-nakin* (Il-1 derivatives) *-leukin* (Il-2 derivatives) *-plestim* (Il-3 derivatives) *-exakin* (Il-6 derivatives)
-kinra	interleukin receptor antagonists; *-nakinra* (Il-1 receptor antagonists)
-kiren	renin inhibitors
-leukin	see *-kin*
-lipastat	see *-stat*
-lukast	see *-ast*
-mab	monoclonal antibodies (for details please see page .....)
-mantadine	adamantane derivatives; *-mantine, -mantone*
-meline	cholinergic agents, arecoline derivatives
-mer	polymers
-mesine	sigma receptor ligands
-mestane	aromatase inhibitors
-metacin	anti-inflammatory, indometacin derivatives
-micin	antibiotics obtained from various *Micromonospora*
-monam	monobactam antibiotics
-morelin	see *-relin*
-mostim	see *-stim*
-motine	antivirals, quinoline derivatives
-moxin	monoamine oxidase inhibitors, hydrazine derivatives**not part of definition
-mustine	antineoplastic, alkylating agents, (b-chloroethyl)amine derivatives
-mycin	antibiotics, produced by *Streptomyces strains*
nab	cannabinol derivatives
-nakin	see *-kin*

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STEM	*DEFINITION* *substem, if available*
-nakinra	see *-kinra*
nal-	narcotic antagonists/agonists related to normorphine
-nercept	tumor necrosis factor antagonist
-nermin	see *-ermin*
nic	nicotinic acid or nicotinoyl alcohol derivatives
-nicate	antihypercholesterolaemic and/or vasodilating nicotinic acid esters
-nidazole	antiprotozoals, metronidazole derivatives
nifur-	5-nitrofuran derivatives
-nixin	anti-inflammatory, anilinonicotinic acid derivatives
-nonacog	see *-cog*
-octocog	see *-cog*
-olol	b-adrenoreceptor antagonists; *-alol*: aromatic ring -CH-CH2-NH-R related to -olols
-olone	steroids other than prednisolone derivatives
-opamine	dopaminergic agents dopamine derivatives used as cardiac stimulant/antihypertensives/diuretics
-onide	steroids for topical use, acetal derivatives
-(o)nidine	antihypertensives, clonidine derivatives
-orex	anoretics
orphan	narcotic antagonists/agonists, morphinan derivates; *-orphine, -orphinol, orphone*
-oxacin	antibacterials, nalidixic acid derivatives
-oxan(e)	benzodioxane derivatives
-oxanide	antiparasitics, salicylanides and analogues
-oxef	antibiotics, oxacefalosporanic acid derivatives
-oxetine	antidepressants, fluoxetine derivatives
-pafant	platelet-activating factor antagonists
-pamide	diuretics, sulfamoylbenzoic acid derivatives
-pamil	coronary vasodilators, verapamil derivatives
-parcin	glycopeptide antibiotics
-parin	heparin derivatives including low molecular mass heparins
-penem	analogues of penicillanic acid antibiotics modified in the five-membered ring
-peridol	see *-perone*
-peridone	see *-perone*
-perone	tranquillizers, neuroleptics, 4'-fluoro-4-piperidinobutyrophenone derivatives; *-peridol* (antipsychotics, haloperidol derivatives); *-peridone* (antipsychotics, risperidone derivatives)
-pidem	hypnotics/sedatives, zolpidem derivatives
-pin(e)	tricyclic compounds; *-apine* (psychoactive); *-cilpine* (antiepileptic); *-dipine (see -dipine)* *-zepine* (antidepressant/neuroleptic); *-oxepin, -oxopine, -sopine, -tepines*
-piprazole	psychotropics, phenylpiperazine derivatives
-pirox	antimycotic pyridone derivatives
-planin	antibacterials (*Actinoplanes* strains)

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STEM	***DEFINITION*** ***substem, if available***
-platin	antineoplastic agents, platinum derivatives
-plestim	see *-stim*
-plon	pyrazolo[.]pyrimidine derivatives, used as anxiolytics, sedatives, hypnotics
	-poetin erythropoietin type blood factors
	-porfin benzoporphyrin derivatives
-pramine	substances of the imipramine group
-prazole	antiulcer, benzimidazole derivatives
pred	prednisone and prednisolone derivatives; *-methasone* or *-metasone, -betasol*
-pressin	vasoconstrictors, vasopressin derivatives
-pride	sulpiride derivatives
-pril(at)	angiotensin-converting enzyme inhibitors
-prim	antibacterials, trimethoprim derivatives
-profen	anti-inflammatory agents, ibuprofen derivatives
prost	prostaglandins;
	-prostil (prostaglandins, anti-ulcer)
-prostil	see *-prost*
-quinil	benzodiazepine receptor partial agonists (quinoline derivatives)
-racetam	amide type nootrope agents, piracetam derivatives
-relin	prehormones or hormone-release stimulating peptides:
	-morelin (growth hormone release-stimulating peptides);
	-tirelin (thyrotropin releasing hormone analogues)
-relix	hormone-release inhibiting peptides
-renone	aldosterone antagonists, spironolactone derivates
-restat (or -restat-)	see *-stat*
retin	retinol derivatives
-ribine	ribofuranil-derivatives of the *pyrazofurin* type
rifa-	antibiotics, rifamycin derivatives
-rinone	cardiac stimulants, amrinone derivatives
-rizine	see *-izine*
-rozole	aromatase inhibitors, imidazole-triazole derivatives
-rubicin	antineoplastic antibiotics, daunorubicin derivatives
sal	salicylic acid derivatives: *-sal-, salazo-, -salazine/-salazide, -salan*
-sartan	angiotensin II receptor antagonists, antihypertensive (non-peptidic)
-semide	diuretics, furosemide derivatives
-sermin	see *-ermin*
-serpine	derivatives of *Rauwolfia* alkaloids
-setron	serotonin receptor antagonists (5-HT_3) not fitting into other established groups of serotonin receptor antagonists
som-	growth hormone derivatives
-spirone	anxiolytics, buspirone derivatives
-stat (or -stat-)	enzyme inhibitors:
	-lipastat (pancreatic lipase inhibitors);
	-restat or *-restat-* (aldose-reducing inhibitors);

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STEM	*DEFINITION* ***substem, if available***
	-vastatin (antilipidemic substances, HMG CoA reductase inhibitors)
-steine	mucolytics, other than bromhexine derivatives
-ster-	androgens/anabolic steroids: *-testosterone, -sterone, -ster-, -gesterone, -sterone, sterol, ster,* *-(a)steride* (antineoplastics)
-stim	colony stimulating factors: *-grastim* (granulocyte colony stimulatory factor (G-CSF) type) substances; *-gramostim* (granulocyte macrophage colony stimulating factor (GM-CSF) type substances); *-mostim* (macrophage stimulating factors (M-CSF) type substances); *-plestim* (interleukin-3 analogues and derivatives)
sulfa-	anti-infectives, sulfonamides
-sulfan	antineoplastic, alkylating agents, methanesulfonates
-tecan	antineoplastics, topoisomerase I inhibitors
-tepa	antineoplastics, thiotepa derivatives
-teplase	see *-ase*
-terol	bronchodilators, phenethylamine derivatives [previously *-prenaline* or *-terenol*]
-terone	antiandrogens
-tiazem	calcium channel blockers, diltiazem derivatives
-tide	peptides and glycopeptides (for special groups of peptides see *-actide, -pressin, -relin, -tocin)*
-tidine	histamine H_2-receptor antagonists, cimetidine derivatives
-tirelin	see *-relin*
-tizide	diuretics, chlorothiazide derivatives
-tocin	oxytocin derivatives
-toin	antiepileptics, hydantoin derivatives
-trexate	folic acid analogues
-tricin	antibiotics, polyene derivatives
-triptyline	antidepressants, dibenzo[*a,d*]cycloheptane or cyclopheptene derivatives
-troban	thromboxane A_2-receptor antagonists; antithrombotic agents
-trodast	see *-ast*
trop	atropine derivatives
-uplase	see *-ase*
-uracil	uracil derivatives used as thyroid antagonists and as antineoplastics
-uridine	uridine derivatives used as antiviral agents and as antineoplastics; also *-udine*
-vastatin	see *-stat*
-verine	spasmolytics with a papaverine-like action
vin- or -vin-	vinca alkaloids
vir	antivirals (undefined group): *-amivir* (neuraminidase inhibitors); *-cavir* (carbocyclic nucleosides); *-virsen* (antisense oligonucleotides)
-virsen	see *vir*

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STEM	***DEFINITION*** ***substem, if available***
-vudine	antiviral; antineoplastics, zidovudine derivatives
-xanox	antiallergic respiratory tract drugs, xanoxic acid derivatives
-zafone	alozafone derivatives
-zepine	see *-pine*

Explanatory note:
The hyphens indicate the position of the stem, prefix, infix or suffix, within the INN. In the event that the hyphen is absent, the stem may be used in any position within the name.

The following common stems have been discontinued:

STEM	***DEFINITION***
mer- or -mer-	mercury-containing drugs, antimicrobial or diuretic (deleted from General Principles in List 28 prop. INN)
mito-	antineoplastics, nucleotoxic agents (deleted from General Principles in List 24 prop. INN)
-ol	alcohols and phenols (deleted from Generla Principles in 14th report)
-quine, quin	quinoline derivatives (deleted from General Principles in List 28 prop. INN)
-stigmine	anticholinesterases (deleted from General Principles in List 24 prop. INN)

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ANNEX 4

Specific groups of biological compounds

Peptides, glycopeptides, proteins and glycoproteins (general approach)

The INN experts have adopted the following general scheme for the naming of peptides/proteins:

1. selection of a *stem* for the main compound, e.g. *-poetin* (for erythropoietin derivatives), *-irudin* (for hirudin derivatives), *-cog* (for blood coagulation factors);
2. designation of *subgroups* by expanding the stem, e.g. *-eptacog, -octacog;*
3. selection of a *random prefix* for compounds with differences in aminoacid sequence;

In addition for *glycosylated compounds:*

4. selection of a Greek letter spelt out as a second part of a two-word name for glycosylated compounds with identical amino acid sequence and different glycosylation pattern.

Blood coagulation factors

The following stems, infixes and suffixes, have been selected up to date for recombinant blood coagulation factors:

blood coagulation factors: *-cog*
- factor VII : *(-)eptacog*
- factor VIII : *(-)octocog*
- factor IX : *(-)nonacog*

A prefix will be necessary if the amino acid sequence does not match that of the naturally occurring material. In accordance with the general policy, *alfa, beta,* etc., will be added for the glycoproteins. When the additional statement "activated" is needed, i.e. for the blood coagulation factor VIIa, it should be spelt out in full and added in parenthesis after the name.

Colony stimulating factors

A general stem for all colony stimulating factors was selected and substems for the various categories were designated:

colony stimulating factors *: -stim*

combination of two different types of colony stimulating factors:	*-distim*
granulocyte colony stimulating factor (G-CSF) type substances :	*-grastim*
granulocyte macrophage colony stimulating factor (GM-CSF) type substances :	*-gramostim*
macrophage stimulating factor (M-CSF) type substances :	*-mostim*
interleukin-3 analogues and derivatives:	*-plestim*

Enzymes

The common stems for enzymes, in general, is *-ase.* Substems are referring to the origin of the substances, e.g. tissue plasminogen activator and urokinase-type plasminogen activators.

enzymes : *-ase*

enzyme with superoxide dismutase activity :	*-dismase*
tissue-type plasminogen activators :	*-teplase*
urokinase-type plasminogen activators :	*-uplase*

Further examples of enzymes may be found in *WHO/PHARM S/NOM 15*

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Erythropoietins

In the case of erythropoietins it was decided to select *epoetin* together with a Greek letter to differentiate between compounds of the same amino acid sequence as human erythropoietin which vary in the glycosylation pattern. INNs with different amino acid sequence will be named using the *-poetin* stem and a random prefix.

erythropoietin type blood factors : *-poetin*

Growth factors

The general stem assigned for growth factors is *-ermin*. Substems allow distinction between the various types of growth factors, for example epidermal growth factors, fibrinoblast growth factors and insulin-like growth factors.

When selecting a name for tumor necrosis factors (TNF) these were also classified as growth factors.

growth factors : *-ermin*

epidermal growth factors :	*-dermin*
fibrinoblast growth factors :	*-fermin*
tumor necrosis factors (TNF):	*-nermin*
platelet-derived growth factor:	*-plermin*
insulin-like growth factors :	*-sermin*
transforming growth factor:	*-termin*

Growth hormones

The characteristic stem for this group of compounds is the prefix *som-*. For substances other than human, suffixes are added to indicate the species specificity of the structure are as follows:

growth hormones: *som-*

bovine-type substances :	*-bove*
porcine-type substances:	*-por*
salmon-type substances :	*-salm*

Hirudin analogues

Hirudin is a well-established name for an anticoagulant isolated from medicinal leeches. Hirudin analogues are non-glycosylated polypeptides produced by recombinant biotechnology. The stem *-irudin* will be used for hirudin compounds, a random prefix will allow to differentiate for different amino acid sequences.

hirudin analogues : *-irudin*

Hormone release stimulating peptides

The common stem selected for hormone release stimulating peptides is *-relin*. INNs for hormone-release inhibiting peptides should include the stem *-relix*.

hormone-release stimulating peptides : *-relin*

growth hormone release stimulating peptides :	*-morelin*
thyrotropin releasing hormone analogues:	*-tirelin*

hormone-release inhibiting peptides : *-relix*

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Interleukins

The first general stem selected for interleukins was *-leukin*, which was derived from the name *interleukin* assigned by the *International Union of Biochemistry* (IUB) - *International Union of Pure and Applied Chemistry* (IUPAC) - *Joint Commission on Biochemical Nomenclature* (JCBN). Randomly assigned prefixes should distinguish between the different compounds.

Based on the needs for naming further interleukins, the following stem system was accepted for recombinant interleukins :

interleukin	*INN stem*
Il-1	*-nakin*
Il-2	*-leukin*
Il-3	*-plestim*
Il-6	*-exakin*
IL-8	*-octakin*
IL-11	*-elvekin*
receptor antagonist:	*-kinra*
Il-1	*-nakinra*

It was agreed to publish the INNs for glycosylated interleukins with *alfa, beta*, etc, in accordance with the general policy for naming glycosylated proteins.

Pituitary hormones

The name selected by the IUPAC-IUB have, to date, been chosen for compounds with identical amino acid sequence as the naturally occurring human hormone. Addition of a Greek letter as second name will allow to differentiate for different glycosylation pattern for compounds produced by biotechnology.

The following scheme is at present in use :

pituitary hormones : *-tropin*
follicle stimulating hormones : *(-)follitropin*
luteinizing hormones : *(-)lutropin*

Monoclonal antibodies

The following scheme for common stems has been developed for naming monoclonal antibodies:

I.	*General stem*:		*-mab*
II.	*Sub-stems for source of product:*		
		human	*-u-*
		rat	*-a-*
		hamster	*-e-*
		primate	*-i-*
		mouse	*-o-*
		chimeras	*-xi-*
		humanized	*-zu-*

The distinction between chimeric and humanized antibodies is as follows:

A chimeric antibody is one that contains contiguous foreign-derived amino acids comprising the entire variable region of both heavy and light chains linked to heavy and light constant regions of human origin.

A humanized antibody has heavy (H) and light (L) chain variable (V) regions, consisting of the amino acids comprising the complementarity-determining region (CDR) segments (and possibly frameword residues)

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from foreign antibodies inserted appropriately among variable regions framework segments of human-derived amino acid residues, linked to H and L constant regions of human origin.

III. *Sub-stems for disease or target group:*

bacterial	*-ba(c)-*
cardiovascular	*-ci(r)-*
immunomodulator	*-li(m)-*
infectious lesions	*-le(s)-*
viral	*-vi(r)-*
tumors:	
colon	*-co(l)-*
testis	*-go(t)-*
ovary	*-go(v)-*
mammary	*-ma(r)-*
melanoma	*-me(l)-*
prostate	*-pr(o)-*
miscellaneous	*-tu(m)-*

Whenever there is a problem in pronunciation, the final letter of the sub-stems for diseases or targets may be deleted, e.g. *-co(l)-, vi(r), li(m),* etc.

IV. *Prefix:*

The prefix should be random, e.g. the only requirement is to contribute to a euphonious and distinctive name.

IV. *Second word:*

If the product is radiolabelled or conjugated to another chemical, such as a toxin, dentification of this conjugate is accomplished by use of a separate, second word or acceptable chemical designation. For monoclonals conjugated to a toxin, the *tox-* stem must be included as part of the name selected for the toxin.

If the monoclonal antibody is used as a carrier for a radioisotope, the latter will be listed first in the INN, e.g. technetium (^{99m}Tc) pintumomab.

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ANNEX 5

WHA46.19 Nonproprietary names for pharmaceutical substances

The Forty-sixth World Health Assembly,

Recalling resolution WHA31.32 on the importance of using nonproprietary names in establishing national drug formularies;

Noting the fundamental contribution of the WHO programme on international nonproprietary names (INN) to effective communication in medicine, and the challenge inherent in maintaining the nomenclature as new substances are introduced into clinical use;

Acknowledging with satisfaction the increasing contribution of generic products to national drug markets in both developed and developing countries;

Noting the current trend to market products with the same active ingredient as, and intended to be clinically interchangeable with, a product currently on the market (multisource products) under trade-marks or brandnames derived from stems or other descriptors for international nonproprietary names nomenclature;

Recognizing that such use, particularly in respect of single-ingredient prescription drugs, may compromise the safety of patients by creating confusion in prescribing and dispensing medicines and by interfering with the orderly development of the nomenclature for international nonproprietary names;

Aware of the concern expressed by the International Conference of Drug Regulatory Authorities at its last meeting about the increasing use of pharmaceutical brandnames that are very similar to or derived from international nonproprietary names;

Noting the recommendation made by the WHO Expert Committee on the Use of Essential Drugs, in its fifth report,[1] on the need to discourage, as a matter of urgency, the use of trade-marks that are derived from international nonproprietary names,

1. REQUESTS Member States:

(1) to enact rules or regulations, as necessary, to ensure that international nonproprietary names (or the equivalent nationally approved generic names) used in the labelling and advertising of pharmaceutical products are always displayed prominently;
(2) to encourage manufacturers to rely on their corporate name and the international nonproprietary names, rather than on trade-marks, to promote and market multisource products introduced after patent expiration;

(3) to develop policy guidelines on the use and protection of international nonproprietary names, and to discourage the use of names derived from INNs, and particularly names including established INN stems as trade-marks;

2. CALLS ON the Director-General to intensify his consultations with governments and representatives of the pharmaceutical industry on ways of reducing to a minimum the problems arising from drug nomenclatures that may create confusion and jeopardize the safety of patients.

Twelfth plenary meeting, 12 May 1993
A46/VR/12

[1] *WHO Technical Report Series*, No. 825, 1992.

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ANNEX 6

Procedure for the selection of international nonproprietary names for pharmaceutical substances

1. Proposals for recommended international nonproprietary names shall be submitted to the World Health Organization on the form provided therefore.

2. Such proposals shall be submitted by the Director-General of the World Health Organization to the members of the Expert Advisory Panel on the International Pharmacopoeia and Pharmaceutical Preparations designated for this purpose, for consideration in accordance with the "*General principles for guidance in devising International Nonproprietary Names*". The name used by the person discovering or first developing and marketing a pharmaceutical substance shall be accepted, unless there are compelling reasons to the contrary.

3. Subsequent to the examination provided for in article 2, the Director-General of the World Health Organization shall give notice that a proposed international nonproprietary name is being considered.

A. Such notice shall be given by publication in the *Chronicle of the World Health Organization*[1] and by letter to Member States and to national pharmacopoeia commissions or other bodies designated by Member States.

(i) Notice may also be sent to specific persons known to be concerned with a name under consideration.

B. Such notice shall:

(i) set forth the name under consideration;
(ii) identify the person who submitted a proposal for naming the substance, if so requested by such person;
(iii) identify the substance for which a name is being considered;
(iv) set forth the time within which comments and objections will be received and the person and place to whom they should be directed;
(v) state the authority under which the World Health Organization is acting and refer to these rules of procedure.

C. In forwarding the notice, the Director-General of the World Health Organization shall request that Member States take such steps as are necessary to prevent the acquisition of proprietary rights in the proposed name during the period it is under consideration by the World Health Organization.

4. Comments on the proposed name may be forwarded by any person to the World Health Organization within four months of the date of publication, under article 3, of the name in the *Chronicle of the World Health Organization.*[1]

5. A formal objection to a proposed name may be filed by any interested person within four months of the date of publication, under article 3, of the name in the *Chronicle of the World Health Organization.*[2]

A. Such objection shall:

(i) identify the person objecting;
(ii) state his interest in the name;
(iii) set forth the reasons for his objection to the name proposed.

6. Where there is a formal objection under article 5, the World Health Organization may either reconsider the proposed name or use its good offices to attempt to obtain withdrawal of the objection. Without prejudice to the consideration by the World Health Organization of a substitute name or names, a name shall not be selected by the World Health Organization as a recommended international nonproprietary name while there exists a formal objection thereto filed under article 5 which has not been withdrawn.

[1] The title of this publication was changed to *WHO Chronicle* in January 1959. From 1987 onwards lists of INNs are published in *WHO Drug Information*.

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7. Where no objection has been filed under article 5, or all objections previously filed have been withdrawn, the Director-General of the World Health Organization shall give notice in accordance with subsection A of article 3 that the name has been selected by the World Health Organization as a recommended international nonproprietary name.

8. In forwarding a recommended international nonproprietary name to Member States under article 7, the Director-General of the World Health Organization shall:

A. request that it be recognized as the nonproprietary name for the substance; and

B. request that Member States take such steps as are necessary to prevent the acquisition of proprietary rights in the name, including prohibiting registration of the name as a trade-mark or tra de-name.

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ANNEX 7

Applications for INNs through national authorities (addresses)

a) National Nomenclature Commissions:

France: **DCF** - Dénominations Communes Françaises

Agence du Médicament
Direction des Laboratoires et des Contrôles
Unité Pharmacopée
145-147 boulevard Anatole France
F-93285 Saint-Denis Cedex
France

Italy: **DCIt** Commission - Denominazione Communi Italiane

Director-General
Pharmaceutical Division
Ministero della Sanità
Viale della Civiltà Romana 7
I-00144 Roma
Italy

Japan: **JAN** - Japanese Accepted Names

Japanese Ministry of Health and Welfare
New Drugs Division
Pharmaceuticals Affairs Bureau
1-2-2, Kasumigaseki, Chiyoda-ku
Tokyo 100
Japan

United Kingdom: **BAN** - British Approved Names

The Secretary
British Pharmacopoeia Commission
Market Towers
1 Nine Elms Lane
London SW8 5NQ
United Kingdom

USA: **USAN** - United States Adopted Names

The Secretary
United States Adopted Names Council
American Medical Association
515 North State Street
Chicago, Illinois 60610
USA

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b) Other national nomenclature authorities:

Belgium:

L'Inspecteur en chef-Directeur
Ministère de la Santé Publique et de l'Environnement
Inspection générale de la Pharmacie
Cité administrative de l'Etat
Quartier Vésale 333
B-1010 Bruxelles
Belgium

China:

The Deputy Chief
Drug Standard Division II
The Chinese Pharmacopoeia Commission
Ministry of Health
Temple of Heaven
Beijing 100050
People's Republic of China

Hungary:

Director-General
National Institute of Pharmacy
P.O. Box 450
1372 Budapest 5
Hungary

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ANNEX 8

WORLD HEALTH ORGANIZATION **ORGANISATION MONDIALE DE LA SANTE**	**Request for an international nonproprietary name (INN)** **Demande de dénomination commune internationale (DCI)**
Authority or manufacturer: *Autorité ou fabricant:* Name of applicant / *nom du demandeur:* Name of responsible officer / *nom du responsable:* Address / *adresse:* Telephone No/*No. de téléphone.* Fax No./*No de fax* We hereby request the World Health Organization to establish a free and unrestricted INN for the pharmaceutical substance described below *L'OMS est priée de bien vouloir établir une DCI à usage libre pour la substance pharmaceutique en question*	**For completion by WHO/** ***A remplir par l'OMS*** No. Date: Copies forwarded: Date: Acknowledged:

SUGGESTED NAMES (in order of preference): 1.....
DENOMINATIONS PROPOSÉES
(par ordre de préférence) 2.....
3.....

CHEMICAL NAME OR DESCRIPTION (INCLUDING STEREOCHEMICAL INFORMATION):
NOM OU DESCRIPTION CHIMIQUE (Y COMPRIS DE L'INFORMATION SUR LA STÉRÉOCHIMIE)

GRAPHIC FORMULA:
FORMULE GRAPHIQUE

MOLECULAR FORMULA:
FORMULE BRUTE

CHEMICAL ABSTRACTS SERVICE (CAS) REGISTRY NUMBER:
NUMÉRO DU REGISTRE CAS

TRADE NAME (known or contemplated):
NOM COMMERCIAL (connu ou envisagé)

ANY OTHER NAME OR CODE:
AUTRE NOM OU CODE

PRINCIPAL THERAPEUTIC USES AND POSOLOGY; PHARMACOLOGICAL ACTION:
UTILITÉ THÉRAPEUTIQUE ET POSOLOGIE; ACTION PHARMACOLOGIQUE

For completion by WHO/
A remplir par l'OMS

WHO 5233* PHA (9/97) - 1000 *PTO/TSVP*

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1. The process of selecting an INN should be initiated during that period of investigation when the compound is undergoing clinical study in human subjects. Please indicate the date when clinical trials began:
La procédure de sélection d'une DCI débute pendant la période d'investigation au cours de laquelle la substance fait l'objet d'études cliniques sur des sujets humains. Veuillez indiquer à quelle date ont débuté les essais cliniques:

2. This proposal is made on the understanding that insofar as is known, none of the suggested names is either registered or pending registration.
En présentant cette proposition, le signataire déclare qu'à sa connaissance aucune des dénominations suggérées n'a été déposée ou n'est sur le point de l'être.

3. The Undersigned confirms that the Chemical Abstracts Service (CAS) number is correct and permission is granted to WHO to publish it in the INN publications.
Le soussigné confirme que le numéro dans le registre du CAS est correcte et que l'OMS est autorisée à le publier dans les publications relatives aux DCIs.

ADDITIONAL COMMENTS:
REMARQUES

Date ..... *Signature* .....

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Safety Issues Specific to Clinical Development of Protein Therapeutics

CA Haller[1], ME Cosenza[1] and JT Sullivan[1]

Discoveries in molecular sciences have advanced research into therapeutics that target an array of newly identified ligands and receptors with the hope of meeting critical needs of patients with chronic or life-threatening diseases. Protein or biologic therapeutics include peptides, endogenous proteins, monoclonal antibodies, hybrids such as Fc construct molecules (including peptibodies and soluble receptors), and avimers. Proteins may be purified animal derivatives, fully human, or have portions of animal and human proteins engineered together into chimeric and humanized monoclonal antibodies. Although some reports distinguish therapeutic proteins from monoclonal antibodies, for the purposes of this article the terms biologics and proteins are used interchangeably.

Although data are not available on other biologics, there are 21 monoclonal antibodies currently approved for use worldwide and more than 200 are in clinical development. The number of protein therapeutics entering clinical trials has increased nearly threefold in the past 10 years.[1] In clinical development, the average duration of time before a biologic product is approved is 7.6 years, comparable to the 7.5 years required for approval of small-molecule drugs. The success rate of humanized monoclonal antibodies entering clinical trials is 17% overall; 15% for anticancer candidates and 21% for immunological candidates, which is slighter higher than the approval rates for small-molecule drugs.

The safety of early clinical studies involving novel therapeutic proteins has come under increased scrutiny since the disastrous First-in-Human (FIH) trial of TGN1412 in 2006, the so-called TeGenero incident. Six volunteer subjects received a CD28 super agonist and experienced an unexpected, severe adverse reaction known as a "cytokine release storm" requiring intensive care management.[2] Species differences in potency, target affinity, and immune response may have been responsible for the discrepancies between the animal and the FIH-dosing experiences with this potent immune system agonist.[3] One outcome of the regulatory investigation that followed this tragedy was the convening of an expert scientific group that issued a number of recommendations to improve the safety of future clinical trials of similar investigational products.[4] In this article, we highlight key safety issues that are of relevance in the clinical development of protein therapeutics, from FIH safety observations to post-marketing surveillance. We focus on novel therapies that may be of potential high risk and necessitate special measures to ensure the well being of research study volunteers as well as patients.

PHARMACOKINETICS/PHARMACODYNAMICS OF THERAPEUTIC PROTEINS

Pharmacodynamic effects of biologics typically do not correlate with pharmacokinetic parameters such as peak plasma concentration (C_{max}) or time to maximum plasma concentration (T_{max}). In contrast to small molecules that tend to have concentration-dependent pharmacodynamic effects (both therapeutic and toxic), biologics tend to lag in clinical activity and frequently have a prolonged duration of action. Given that significant exposure to the biologic is often a requisite for attaining a therapeutic effect, area under the plasma concentration time curve (AUC) and trough concentrations are better predictors of pharmacodynamic response (as compared with C_{max}).[5] Certain adverse effects of biologics take time to manifest; for example, the time to onset of skin toxicity after panitumumab is administered averages ~12 days.[6] However, rapid adverse reactions to biologics, such as infusion reactions and cytokine release phenomena, are probably C_{max}-related events.

ON-TARGET TOXICITIES OF THERAPEUTIC PROTEINS

Because biologics are designed to act on highly specific targets and bind extracellularly, off-target activity is generally considered to be uncommon. In contrast, small molecules have direct intracellular effects and frequently exhibit off-target activity such as hepatotoxicity and other effects that are distinct from the desired target. With biologic agents, adverse reactions are most commonly a result of exaggerated pharmacology. Some well known on-target toxicities of selected biologics are listed in Table 1. For example, the cancer therapeutic bevacizumab

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Table 1 Known "on-target" adverse effects of selected protein therapeutics

Target	Generic name(s)	Molecule type	US-approved indication(s)	Observed toxicities
α4 Integrin	Natalizumab	Humanized mAb	Multiple sclerosis; Crohn's disease	Infections including PML; anaphylaxis
CD11a	Efalizumab	Humanized mAb	Psoriasis	Infection; malignancy; immune thrombocytopenia; hemolytic anemia
CD20	Rituximab	Chimeric mAb	NHL; RA	PML; tumor lysis syndrome; severe mucocutaneous reactions
EGFR	Cetuximab	Chimeric mAb	Cancer	Skin toxicity
	Panitumumab	Fully human mAb	Cancer	Skin toxicity
ESA	Darbopoetin-α	Modified growth factor	Anemia	Cardiovascular/thrombotic events
G-CSF	Pegfilgrastim	Pegylated growth factor	Leukopenia	Increased alkaline phosphatase; bone pain
HER-2	Trastuzumab	Humanized mAb	Cancer	Cardiac failure
IgE	Omalizumab	Humanized mAb	Asthma	Anaphylaxis, malignancy
TNF-α	Adalimumab	Fully human mAb	RA; AS; psoriatic arthritis; psoriasis; Crohn's disease	Serious infection
	Etanercept	Fc fusion (soluble receptor)	RA; AS; psoriatic arthritis; psoriasis	Serious infection
	Infliximab	Chimeric mAb	RA; Crohn's disease	Serious infection; hepatosplenic T-cell lymphoma
VEGF	Bevacizumab	Humanized mAb	Cancer	Thromboembolism; hemorrhage; GI perforation; hypertension; wound healing complications; RPLS

AS, ankylosing spondylitis; EGFR, epidermal growth factor receptor; ESA, erythropoiesis stimulating agent; G-CSF, granulocyte colony stimulating factor; GI, gastrointestinal; HER-2, human epidermal growth receptor 2; IgE, immunoglobulin E; mAb, monoclonal antibody; NHL, non-Hodgkins lymphoma; PML, progressive multifocal leukoencephalopathy; RA, rheumatoid arthritis; RPLS, reversible posterior leukoencephalopathy syndrome; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

targets vascular endothelial growth factor and inhibits angiogenesis. The pharmacology of vascular endothelial growth factor inhibition clinically translates into adverse reactions such as hypertension, hemorrhage, and arterial and venous thrombosis.[7] These effects would be considered to be on target. Similarly, skin reactions are common with epidermal growth factor receptor agents such as cetuximab[8] and panitumumab.[9] Dermal effects of these monoclonal antibody oncology products range from common mild rashes to rare, life-threatening skin infections with sepsis. Another example is an increase in unusual infections such as tuberculosis seen with tumor necrosis factor antagonists or sequestering agents; these effects relate to immune modulation activity and are generally considered on target for this class of agents. Some on-target toxicities are more common in patients with predisposing conditions. For example, cardiac toxicity was identified at an unexpectedly high rate in early clinical trials with trastuzumab.[10] Subsequent studies showed that concomitant use of anthracyclines and low baseline left ventricular ejection fraction were predictive of trastuzumab cardiac toxicity.[10]

OFF-TARGET TOXICITIES OF THERAPEUTIC PROTEINS

The most common toxicity associated with biologic agents that could reasonably be considered off target is the phenomenon of injection-site reactions. These occur with most large molecules when they are administered subcutaneously. The redness, swelling, itching, and pain at the site of injection are not generally considered as being on target but as being part of a reaction to a foreign protein. Similar effects are occasionally encountered at the site of an injection administered intravenously.

In addition to local effects at the site of administration, off-target systemic effects are also seen after intravenous administration. These infusion reactions may be related to cytokine release or formation of anti-drug antibodies. Signs and symptoms consist of fever, chills, nausea, fluctuations in heart rate and blood pressure, and skin rash and are most commonly seen after the first infusion. These reactions are more common with chimeric as opposed to humanized or fully human monoclonal antibodies. Generally, the greater the human sequence homology of the antibody, the less the acute reaction. For example, cetuximab (chimeric) is associated with a greater incidence and severity of infusion-related toxicity than is bevacizumab (humanized) or panitumumab (fully human).[12,13] In some cases it is unclear whether infusion reactions are on or off target, because cytokine release may be part of the desired targeted therapeutic response. Accordingly, whether a particular infusion reaction is to be considered on or off target relates to the particular mechanism of action of a given molecule.

Related to, but distinct from, these infusion reactions are anaphylactoid reactions that may be caused by biologic agents. Anaphylactoid reactions are clinically indistinguishable from immunoglobulin E–mediated anaphylactic reactions and may occur after administration of agents such as stem cell factor (Stemgen/Ancestim), when mast cells are released during the mobilization of stem cells from bone marrow.[14] The symptoms include urticaria, angioedema, bronchospasm, and hypotension. Anaphylactic or type 1 hypersensitivity reactions have a similar clinical presentation but a different time course (i.e., they occur most commonly not during the first infusion but after subsequent infusions) and can also occur after subcutaneous administration. Severe systemic immune reactions to biologic agents are less common today because of the use of highly purified recombinant products rather than the bacteria- and

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animal-derived proteins that were formerly manufactured.[15] However, the occurrence of anaphylactic reactions to first-time use of cetuximab was recently linked to pre-existing immunoglobulin E antibodies to the drug in patients' sera.[16]

Another immunogenic reaction is the formation of neutralizing antibodies against the therapeutic protein. The most common effect of the formation of neutralizing antibodies is reduction in the therapeutic efficacy of the drug in the patient (such as human antibody against human antibody). Less often, neutralization of the native substrate occurs, resulting in the opposite of the intended therapeutic effect—shutting down endogenous target activity, sometimes permanently (e.g., pure red-cell aplasia, discussed later). This was also observed during clinical trials of megakaryocyte growth and development factor, a polyethylene glycol–conjugated N-terminal domain of thrombopoietin, which is no longer in clinical development. Subjects developed neutralizing antibodies against megakaryocyte growth and development factor, and these also neutralized endogenous thrombopoietin, rendering these individuals unable to generate platelets.[17,18] Although fully human proteins tend to be less immunogenic than nonhuman proteins, as mentioned earlier, on rare occasions close homology to endogenous proteins may elicit antibody formation and attacks on the host substrate, with devastating effects.

BIOSIMILARS/BIOGENERICS

Adverse effects of biologics that are first observed in the postmarketing period may be related to process or formulation variability due to manufacturing differences. In contrast to generic drugs, in which the active pharmaceutical ingredient is identical in composition to the patented small molecule of its type, so-called biosimilars or biogenerics can differ significantly from the innovator product. The production of protein therapeutics is a highly complex biotechnological process that requires superior sterility, handling, storage, and quality control. Improper formulation of the molecule can result in aggregation, conformational change, or oxidation, which could render the product more immunogenic or less efficacious.[19] Impurities or contaminants can themselves be immunogenic or may increase the immunogenicity of the therapeutic protein.

One example is the increased incidence of pure red-cell aplasia observed in the early 2000s associated with the use of a particular recombinant erythropoietin called Eprex (Johnson & Johnson, Bridgewater, NJ)[20] but not with other erythropoietin products marketed at the time. This outbreak involved approximately 200 patients with chronic kidney disease treated with human serum albumin-free epoetin-α via subcutaneous injection. Although the precise cause of this serious adverse reaction has not been clearly identified, the reports of such incidents decreased dramatically after stringent changes were made in manufacturing operations, handling, storage, and administration of the drug. The origin may have been multifaceted, with a number of variables contributing to a formulation change significant enough to induce an immune reaction in patients. Some cited variables include a switch to polysorbate 80 as a stabilizer (instead of human serum albumin), use of rubber-coated syringe stoppers, self-administration by subcutaneous injection (rather than intravenous administration), and inadequate cold storage.[21] Recent biophysical studies of two epoetin-α products, Epogen (Amgen, Thousand Oaks, CA) and Eprex, revealed that the proteins are not identical, providing solid evidence that biologics marketed as the same drug can be fundamentally different.[22]

LIFE CYCLE SAFETY MANAGEMENT

As with small molecules, with biologics rare serious adverse events may not be detected until a large number of patients have been exposed, frequently in the early postmarketing period. Although rare off-target, idiosyncratic-type reactions can occur with biologics, many serious adverse events are on-target effects such as progressive multifocal leukoencephalopathy associated with the use of the immune system modulators natalizumab[23] and rituximab.[24] Such events could be predicted based on known mechanisms and targets of these novel therapeutics, but they may not be detected in preclinical studies and clinical trials because of the small numbers of treated animals and subjects, respectively.

For products that pose an unusual type or high level of risk, a risk-minimization plan known as Risk Evaluation and Mitigation Strategy may be required for US regulatory filing of a new drug application to minimize risks to patients. This process utilizes several elements to ensure safe use and may include product labeling, targeted education and outreach, the requirement that prescriptions be issued only by trained specialists, informed patient consent, restricted distribution, and restricted-use programs such as performance-linked access systems.[25] In the European Union, all new drug candidates (not only products with identified risks) must have a risk-management plan prior to review.

THERAPEUTIC PROTEINS OF POTENTIALLY HIGH RISK

After the occurrence of adverse reactions in the TGN1412 trial, the Expert Scientific Group was established by the European Medicines Agency to review the lessons learned from this incident and to make recommendations to improve the safety of subjects in future FIH trials involving similar medicines. A key recommendation was to incorporate additional safety measures in the design of early clinical studies of therapeutics of potential high risk.[4] These include products that:

- Have a novel target or mechanism of action for which no previous human data exist;
- Are highly species-specific and for which animal studies may not be predictive or for which responses may be less than anticipated in humans because of differences in target distribution;
- Are highly potent and interact with cellular receptors with greater affinity or in a different way than native ligands; for instance, monoclonal antibodies may cross-link and bind to more than one cell or to other antibodies and produce exaggerated or unintended effects;
- Target the immune system or other highly regulated physiological processes such as endocrine-system cycles or the coagulation cascade.

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The development of protein therapeutics in one or more of these high-risk categories necessitates a more cautious approach to first human exposure.

The Expert Scientific Group recommended several strategies to minimize possible harm from administering initial doses of potentially high-risk products to humans. These include exploring options other than the "no observed adverse effect level" method for dose calculation, such as the "minimal anticipated biological effect level" approach and the use of the lowest effective dose arrived at by various methods of calculation. The "sentinel subject" approach is a prudent strategy that involves sequential dose administration to the first few subjects with an interval of close observation between individuals to identify untoward effects. Selection of the FIH study population also requires careful consideration of the risk:benefit ratio. Healthy volunteers are frequently utilized in FIH trials because they are easier to recruit and have fewer confounding factors such as disease and concomitant medications. With biologics, however, there are instances when selecting a patient population is preferable. Proteins frequently have a long half-life and a prolonged effect lasting weeks or more that may be unacceptable in healthy volunteers. For example, biologics that modulate immune function may be better tolerated by patients with immune disorders such as lupus than by subjects with healthy immune responses. Finally, although clinical benefit is not an expected outcome in most FIH trials, some novel protein therapies may offer promise of relief to patients with grave illnesses for whom few or no treatment options exist.

No drug is without risk. However, most adverse effects of therapeutic proteins are extensions of the known pharmacological actions of the product and therefore can be anticipated and managed. Risks may be titrated to a low level with a thorough understanding of the disease and the molecular target of the therapeutic protein and by careful and prudent dose selection and close patient monitoring. New tools such as genetic screening may be useful in identifying patients who are likely to respond to treatment and in whom some risk is acceptable.[26] Risk evaluation and management should be planned and conducted for the entire life cycle of the medicine. A rational therapeutic approach that matches the right biologic to a suitable patient population minimizes the risk to all who may benefit from the therapeutic protein.

CONFLICT OF INTEREST

All of the authors are employed by Amgen, Inc., a company involved in the discovery, development, manufacture, and sales of protein therapeutics.

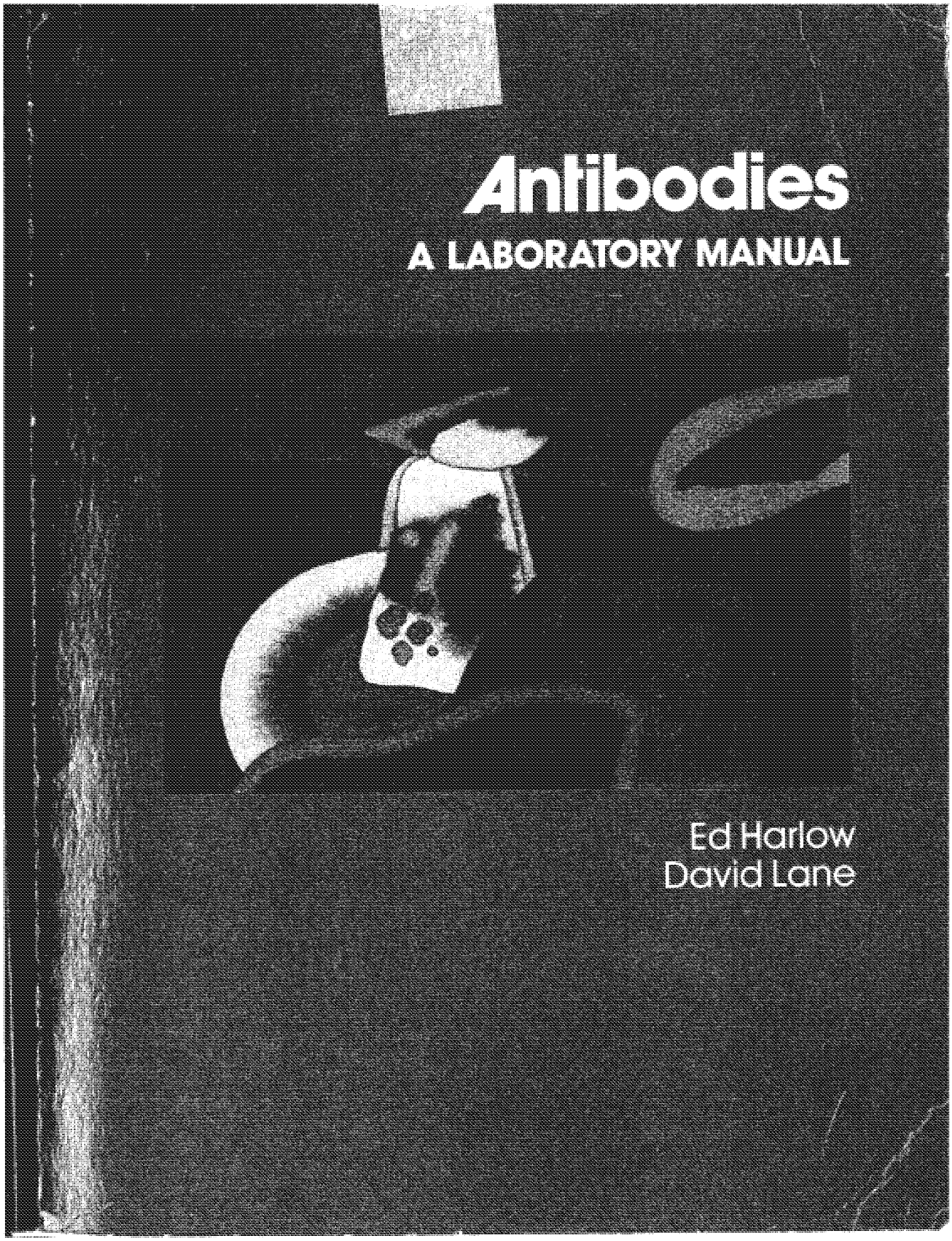
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Antibodies

A LABORATORY MANUAL



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David Lane

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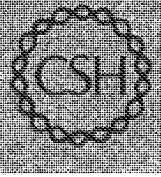
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Antibodies

A LABORATORY MANUAL

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Cold Spring Harbor Laboratory
1988

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Protein G

Protein G is a 30,000- to 35,000-dalton protein isolated from the cell wall of β-hemolytic streptococci of the C or G strains (Kronvall 1973). Less is known about protein G than about protein A, primarily because protein G was not purified until 1984 (Björck and Kronvall 1984). The interest in protein G and in other less well-characterized bacterial cell wall proteins is that these proteins have different affinities for antibodies compared to protein A. Many antibodies from species or subclasses that do not bind well to protein A bind very well to protein G. Tables 15.1 and 15.2 summarize the affinity of protein G for a spectrum of antibody sources.

Like protein A, the gene for protein G has been cloned recently, and protein G is now available in purified form, isolated from bacterial overexpression systems. This should allow more convenient applications and should speed the collection of information on its use in immunochemical tests. One disadvantage concerning protein G is that it contains a second binding site that will interact with albumin (Björck et al. 1987). Recently, the antibody binding domain has been separated from the albumin domain by genetic manipulations, and this should increase the usefulness of protein G.

ANTI-IMMUNOGLOBULIN ANTIBODIES

When purified antibodies from one species are injected into a distant species, the antibodies are often recognized as foreign antigens and elicit a strong humoral response. This characteristic can be used to generate specific anti-immunoglobulin reagents. Using anti-immunoglobulin antibodies solves many of the difficulties in locating and quantitating antibodies when these molecules are found in complex mixtures of serum or tissue culture proteins. The specificity of the anti-immunoglobulin antibodies can be used in many of the same ways and with many of the same advantages as anti-antigen antibodies. One of the major advantages of using these anti-immunoglobulin reagents is that with careful preparation they can be made species, class, subclass, or domain specific. Therefore, anti-immunoglobulin antibodies are prepared for two purposes, either as general reagents used to bind all antibodies or as selective reagents used to identify one type of antibody molecule.

Using antibodies as immunogens has proven to be one of the best experimental systems for studying how a region of a protein is recognized as foreign and how it elicits a humoral response. In practical terms, if a region of the injected antibody molecule is identical to or is sufficiently similar to the corresponding region of the antibodies from the host animal, this region will not be seen as foreign and will not elicit a response. Therefore, the regions that can be recognized as foreign in the generation of anti-immunoglobulin antibodies are determined by the genetic differences between the animal from which the immunogen is prepared and the immunized animal. In addition, as the antibodies become more dissimilar, the number of regions that will be recognized as foreign increases.

In general, animals from different genuses will respond to a number of epitopes on both the heavy and light chains, and these immunogens will induce a strong humoral response. The epitopes recognized in these types of immunizations are described as *xenotypic*. Immunoglobulins isolated from one strain of a species and injected into another strain will induce a response to *allotypic* epitopes. When immunoglobulins are isolated from one inbred strain and injected into the same strain, the epitopes that are recognized are known as *idiotypic* epitopes. Idiotypic epitopes are associated with the hypervariable regions that form the antigen combining site of a particular antibody (see Chapters 2 and 3). Idiotypic epitopes are a subset of the allotypic epitopes which in turn are a subset of the xenotypic epitopes.

One important aspect in the generation of an anti-immunoglobulin response that is not obvious from the above discussion is how similar antibodies are from species to species. This becomes apparent when analyzing the cross-reactions of anti-immunoglobulin antibodies. Although a region may be seen as foreign between one species and another (i.e., the region is different), this does not mean that the same region is not conserved in other species. For example, in a high-titered rabbit anti-mouse immunoglobulin serum, as many as 10% of the antibodies will cross-react with human antibodies. In many instances, these cross-reactions will not lead to any technical problems, but in some cases, such as staining tissue sections, these cross-reactions will ruin an experiment. These types of cross-reactions can be found at all levels between different subclasses, classes, light chains, and species. Eliminating these cross-reactions is one of the problems in preparing antibodies that are specific for different species, different regions of the antibody molecule, or different types of heavy or light chain. To isolate antibodies with these specificities, three steps may be taken. First, the immunization should be targeted to the desired region or polypeptide chain by only injecting this portion of the antibody. Second, the resulting antibodies must then be depleted of all the cross-reacting antibodies by adsorption on samples of the cross-reacting reagents. The last step is to purify the antibodies by binding to the region of interest and then eluting the purified specific antibodies (p. 313).

PREPARING ANTI-IMMUNOGLOBULIN ANTIBODIES

The major consideration when preparing anti-immunoglobulin antibodies is the host in which the antibodies will be raised. This decision should be made on the ease of using the resulting antibodies. In general, although antibodies can be raised in closely related species, most immunizations are done in distant species. Unless there are extenuating circumstances, larger animals yield more sera and should be used if possible. Most of these immunizations are done in rabbits, goats, or sheep. Of these, if protein A will be used in any of the assays, only rabbits produce antibodies that bind protein A with high affinity and are, consequently, the animal of choice. Rabbits are commonly used to prepare anti-mouse, -rat, and -human immunoglobulin antibodies, while goat, sheep, or pigs are used to produce anti-rabbit immunoglobulin antibodies.

Anti-immunoglobulin antibodies are available commercially, and unless large quantities are needed or special antibody couplings are planned, it is seldom worthwhile to prepare these reagents yourself. However, since they are relatively easy to prepare, a suitable protocol is outlined below.

1. Prepare a large batch of antibodies. See p. 288 for appropriate purification protocols. Dissolve the immunoglobulins at approximately 2 mg/ml in PBS.

2. At the time of each injection, make an emulsion of 500 μl of the immunoglobulin solution with 500 μl of Freund's adjuvant. The first injection should be done with complete Freund's; all subsequent injections should be done with incomplete.

   The emulsion can be prepared by taking the 500 μl of each suspension into separate 1-ml syringe barrels. These two solutions are immiscible but with mixing will form an emulsion. This is most easily done by pushing the mixture between the two 1-ml syringes via a two- or three-way valve as shown in Figure 6.5, p. 158). Remove all air from both syringes before connecting to the valve. Make sure the connections are tightened securely. Depress the plunger on the syringe containing the aqueous antigen solution first. Continue to push the mixture between the two syringes until it becomes difficult (approximately 10–20 times). Push the emulsion into one syringe. Remove the valve and the other syringe, and place a 23-gauge needle on the syringe. Displace any air that may be held in the barrel or in the needle by carefully depressing the plunger until the emulsion reaches the needle.

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3. Inject the emulsion into multiple subcutaneous sites in a female New Zealand white rabbit. For most purposes, several rabbits should be injected at the same time to lessen the impact of animal-to-animal differences.

4. Take sample test bleeds from the rabbits 14 days after each injection. Compare the titers using one of the standard quantitative tests or in the tests in which they will be used.

5. Let the rabbits rest for 6 weeks between injections.

6. When the titers have increased to usable levels, each rabbit should be bled to obtain approximately 50 ml of blood. Continue to repeat the injections while collecting the serum 14 days after each injection.

7. Rabbits can continue to produce good anti-immunoglobulin antibodies for over a year. When the last boost has been made, sacrifice the rabbit and collect all the blood. The final bleed will yield approximately 100 ml of serum.

NOTES

i. For protocols in each of the steps above, consult Chapter 5.

ii. This protocol can be modified to produce antibodies that are specific for the antibodies from a particular species, class, or subclass, the Fc or Fab region, or the particular light chain. Begin the immunizations with the desired region or polypeptide chain. After a good immune response has developed, the resulting antibodies are first adsorbed against other regions or polypeptides to remove cross-reacting activities. Then the antibodies are affinity-purified on the starting immunogen (p. 313).

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The Contribution of Contact and Non-contact Residues of Antibody in the Affinity of Binding to Antigen

The Interaction of Mutant D1.3 Antibodies with Lysozyme

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We have analysed the contribution of residues of the D1.3 Fv fragment to binding of hen egg lysozyme. We altered residues at the contact interface by site-directed mutagenesis, and determined the affinity of the mutant Fv fragments for lysozyme by fluorescence quench titration. We found that a band of residues at the centre of the contact interface were much more important for binding affinity than those at the periphery. We also subjected the scFv fragment to random mutagenesis to simulate somatic mutation and affinity maturation. By display of the mutants on the surface of filamentous phages, and selection of the phages with biotinylated lysozyme, we were able to select mutants with modest improvements in binding affinity to lysozyme. By combining the mutations we obtained a scFv fragment with a fivefold improved affinity ($K_d \sim 0{\cdot}6$ nM compared to wild-type $K_d \simeq 3{\cdot}3$ nM). However, none of the altered residues leading to improved affinity was located in the contact interface. This indicates that the interactions of a few residues at the centre of the contact interface are responsible for the binding affinity to antigen, but that these interactions can be modulated by alterations of residues outside the binding site. This may represent a typical mechanism for the affinity maturation of antibodies.

Keywords: antigen binding; affinity maturation; filamentous phage; mutagenesis

In vivo, antibodies are derived by selection from repertoires of antigen-binding sites displayed on the surface of a population of B-cells. The rearranged V-genes, encoding the heavy and light chain variable domains responsible for binding to antigen are created by combinatorial assembly of V-gene elements (heavy chain VH, D and JH; light chain Vλ and Jλ, or Vκ and Jκ) during lymphocyte development. Binding of antigen and help from T-cells triggers differentiation of the B-cell to plasma cells and production of soluble antibody (for review, see Kocks & Rajewsky, 1989; Vitetta *et al.*, 1989). In the mouse, there are approximately $2{\cdot}5 \times 10^8$ B-cells (for review, see MacLennan & Gray, 1986) displaying a large repertoire of approximately 10^7 antigen binding sites (Winter & Milstein, 1991). Each antigen binding site is selected from this large repertoire of structures and, typically, the antigen binding affinities obtained are in the range 10^5 to 10^7 M^{-1} (Winter & Milstein, 1991). The affinities can be improved by random mutation, by which variants are created with an estimated mutation rate of 10^{-3} to 10^{-4} point mutations per base-pair per generation (Griffiths *et al.*, 1984; Berek & Milstein, 1987; Cumano & Rajewsky, 1986; Allen *et al.*, 1987), and those with higher affinity of binding are selected on further immunization with antigen. "Affinity maturation" often leads to improvements in affinity of five- to tenfold (Cumano & Rajewsky, 1986; Berek & Milstein, 1987; Foote & Milstein, 1991).

Although the recognition of antibodies and antigen is based on random encounter, the selected antibody-antigen complexes reveal extensive and highly complementary surfaces with multiple contacts, involving several hypervariable loops of the antibody (for review, see Poljak, 1991). To help dissect the energetics of this recognition, we have exploited the X-ray crystallographic structures of the D1.3 antibody (Amit *et al.*, 1986; Bhat *et al.*, 1990; Fischmann *et al.*, 1991). The monoclonal antibody D1.3 is derived from the secondary immune response (made by immunizing a Balb/c mouse 3

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times over a period of 36 days; Harper *et al.*, 1987). Close germline genes are known (VH = PJ14 (Sakano *et al.*, 1980) and Vκ = K2 (Nishioka & Leder, 1980)) suggesting that D1.3 contains five somatic mutations: Ser56 → Asn in VH CDR2 and Asn50 → Tyr, Ala51 → Thr, Lys52 → Thr in Vκ CDR2 and also a framework mutation Gln66 → His in FR3 of the VH. The Fab and Fv fragments have been solved free, and in complex with hen egg lysozyme: only one of the somatically mutated residues makes direct contact to antigen (VL Tyr50). Here we first tried to identify the contribution of the contact residues defined by X-ray crystallography to the affinity of binding of the Fv fragment.

The contacts between D1.3 Fv or Fab fragments in complex with lysozyme that were analysed are noted in Table 1, and mutants of the Fv fragment were constructed by site-directed mutagenesis. (However, the importance of the main-chain contacts from residues VH 31, VH 53 and VL 91 is impossible to analyse by site-directed mutagenesis.) The binding affinities for lysozyme were measured by fluorescence quench titrations, and are summarized in Table 1. This shows that the residues of loops VH CDR2 and 3 and VL CDR1 and 3 are more important for binding than VH CDR1 and VL CDR2. Residues VH Asp54 and Tyr101 and VL Tyr32 are particularly important, and with VH Trp52 and VL Trp92 help form a central band of key contacts, mainly from aromatic side-chains (Fig. 1). Such contacts have been proposed as major determinants of affinity (Mian *et al.*, 1991), and here form binding pockets, setting up multiple, perhaps co-operative interactions, for example around Gln121 and Asp119 of lysozyme (Amit *et al.*, 1986).

Much of the energetics of interaction seem to be driven by the contacts from a small linear epitope, Gly117 to Gln121 of lysozyme to this central band of residues (HEL Gln121 to VH Tyr101, VL Tyr32 and Trp92; HEL Val120 to VH Tyr 101; HEL Asp119 to VH Trp52 and Tyr101; HEL Thr118 to VH Trp52 and Asp54; and HEL Gly117 to Asp54). Other contacts analysed (VH Thr30, Tyr32 and Arg99, VL Tyr50, Thr53 and Ser93) are less important and are located at the periphery of the antibody binding site (Fig. 1). Each of these peripheral side-chains makes only a single contact with antigen, whereas each residue of the central band makes several. Indeed the number of contacts (clearly related to the location at the centre or periphery) appears to be at least as reliable a guide to predicting the energetics of the interaction of the D1.3 antibody and lysozyme as semi-empirical calculations (Novotny *et al.*, 1989). The effects of the mutation of VH D100 and VH R102 are more difficult to analyse. From the structure, these side-chains seem to make a salt-bridge between each other, and the side-chain of R102 seems to make a hydrogen bond to the main-chain of residue VH 99. Thus alteration of these residues may change the structure of the VH-CDR3 loop.

The dissociation constant of the D1.3 antibody (or Fv fragment) for lysozyme has been determined as 3 to 5 nM by both fluorescence quench titrations (Ward *et al.*, 1989, Foote & Winter, 1992) and titration calorimetry (R. Poljak, personal communication). The affinity of binding is characteristic of a good antibody from the secondary immune response (Foote & Milstein, 1991; Cumano & Rajewsky, 1986), and we wondered if the interaction of these highly intermeshed surfaces could be improved further by random point mutation, and if so whether the contact interface would be altered.

In earlier work, we showed that the affinity of a primary response anti-hapten antibody (B1-8) for

Table 1

Affinity determinations by fluorescence quench titration of mutants of the contact residues

Heavy chain	K_d(nM)	Light chain	K_d(nM)
CDR1			
T30A†	4·3 (0·9)		
Y32F	7·5 (1·8)	Y32F	109·0 (16)
CDR2			
W52A	30·0 (5)	Y50F	15·0 (2)
D54A	183·0 (12)	T53A	2·5 (0·8)
CDR3			
R98A†	8·2 (2·3)	W92A	68·0 (12)
D100A	No binding	S93A	4·5 (1·2)
Y101F	203 (25)		
R102A	No secretion		

Wild-type Fv D1.3 K_d = 3·7 (0·8).

Additional mutations were made in other contact residues (K_d in nM). VH G31A (3·2); VH G53A (43); VL F91Y (22) but the effects are difficult to interpret as these residues are thought to make main chain contacts only. The Fv fragment (version 1) made during the construction of general bacterial expression vectors (Ward *et al.*, 1989) and crystallized by Boulot *et al.* (1990) was used for the site-directed mutagenesis. It was initially subcloned into pUC119 (Vieira & Messing, 1987) to facilitate production of single stranded DNA for sequencing. Site-directed mutagenesis employed the method described by Hemsley *et al.* (1989). In brief, separate vectors were made containing either the heavy or the light chain cassette as it was found to be impossible to perform inverse PCR on a vector containing repeat pelB sequences. Mutants were made to change all the contact residues between D1.3 and HEL (Amit *et al.*, 1986; Bhat *et al.*,1990; Fischman *et al.*, 1991). PCR primers were made to replace tyrosine by phenylalanine and other amino acids by alanine. Putative mutants were sequenced using the dideoxy method (Sanger *et al.*, 1977) and with a Sequenase kit (USB, Cleveland, OH), and after identification of a clone with the correct mutation (and no other PCR-induced errors), the mutant chain was recloned with the other chain to make an Fv construct. Single-stranded DNA was prepared and sequenced again to confirm the desired mutation. The Fv fragments were expressed in bacteria as previously described (see Hawkins & Winter, 1992) and the culture supernatant applied to a 2 ml HEL-Sepharose column, washed with 10 ml phosphate-buffered saline, then 10 ml 100 mM Tris·HCl (pH 8·5), 500 mM NaCl and finally eluted with 5 ml 100 mM triethylamine. The eluted scFv was collected in 1 ml fractions into 0·5 ml ice-cold 1 M Tris·HCl (pH 7·4) and then the peak protein fractions dialysed into phosphate-buffered saline. Affinity measurements were performed by fluorescence quench titration using HEL as described (Foote & Winter, 1992) with ubiquitin added to a final concentration of 25 μg/ml. The results shown are obtained from the mean of 3 or more determinations and the figures in parentheses are standard errors.

† Residues were thought to contact the antigen in lower-resolution structures (Amit *et al.*, 1986) but not in higher-resolution structures (Bhat *et al.*, 1990; Fischmann *et al.*, 1991).

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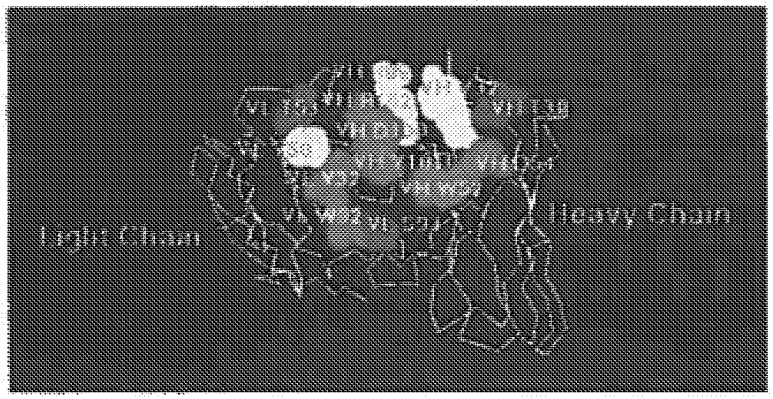


Figure 1. Contact residues of the D1.3 antibody. Conservative mutations in the residues shown in red produce > 8-fold decrease in affinity, those shown in yellow produce a 2 to 8-fold decrease in affinity and those in green < 2-fold decrease in affinity.

the hapten 4-hydroxy-5-iodo-3-nitrophenyl acetate (NIP†) could be matured *in vitro*. We randomly mutated the genes encoding the B1-8 scFv fragment by the polymerase chain reaction (PCR); displayed the population of mutant scFv fragments on filamentous phage; bound the phage to biotinylated NIP-bovine serum albumin and captured the bound phage by streptavidin-coated magnetic beads. This led to the selection of phage bearing antibody fragments with improved affinities of binding to hapten (Hawkins *et al.*, 1992). The affinity improvement obtained was typical of that obtained by the immune system going from the primary to the secondary response (Hawkins *et al.*, 1992; Cumano & Rajewsky, 1986). Here we used the same process of "affinity selection" to isolate mutants of the D1.3 scFv fragment with improved affinity for lysozyme. A library of 2×10^6 members, with an average error rate of 3·8 nucleotide changes per D1.3 scFv gene (42 mutations from 11 random clones sequenced) was displayed on filamentous bacteriophage (McCafferty *et al.*, 1990). After 13 rounds (3 "single" and 5 "double" rounds) of affinity selection, eight mutant clones were sequenced and found to be identical (mutant M1), with residue changes confined to the VH domain (Table 2).

We also deployed the process of "kinetic selection", in which phage with slower dissociation kinetics were selected by loading with biotinylated lysozyme, flooding with unlabelled lysozyme, and then capturing the phage after several lifetimes of the complex (Hawkins *et al.*, 1992). As kinetic selection should be more powerful at discriminating between phage with similar off-rates, mutants were sequenced after five rounds. Of eight mutant clones, five were identical (mutant M2), with residue changes confined to the V_K domain. The other three mutants contained only silent mutations. A further mutant scFv fragment (M3) was constructed by combining the M1 VH domain with the M2 V_K domain (Table 2).

† Abbreviations used: NIP, 4-hydroxy-5-iodo-3-nitrophenyl acetate; PCR, polymerase chain reaction; HEL, Hen egg lysozyme; CDR, complementarity determining region; scFv, single chain Fv. The residues are numbered sequentially from the N terminus for both VH and VL.

Table 2
scFvD1·3 mutants selected from phage repertoires

	Amino acid changes in mutants					
	Heavy chain			Light chain		
	46	58†	63	3	28†	97†
M0	P	D	N	E	I	T
M1	L	A	D	.	.	.
M2	.	.	.	K	T	S
M3	L	A	D	K	T	S

Phage display libraries of random mutants of the scFv D1.3 were made as described (Hawkins *et al.*, 1992). In brief the procedure is as follows: Random mutagenesis was based on the method previously described (Hawkins *et al.*, 1992), except mutating both chains simultaneously using primers VHBBACK (5'-AGG TSM ARC TGC AGS AGT CWG G-3') (Ward *et al.*, 1989) and NotVkFORA (5'-GAT ATG AGA TAC TTG CGG CCG CCG TTT GAT TTC CAG CTT GGT GCC-3'). The product from a 100 µl reaction was extracted once with phenol/chloroform, precipitated with ethanol and then resuspended in 60 µl of water. The vector, Fd.Tet.DOG1, and the PCR product were both digested with *Pst*I (NBL) and *Not*I (NBL) and then gel-purified and the ligation performed.

Biotinylated HEL prepared as described (Hawkins *et al.*, 1992) was used for the selections. The affinity and off-rate selections were performed essentially as described (Hawkins *et al.*, 1992). For affinity selection, the initial HEL concentration used was 2 nM and this was gradually reduced to 0·5 nM as the rounds of selection progressed. For off-rate selection, the concentration of HEL used was 1 nM and with capture 20 min after loading with unlabelled antigen. The binding of the scFvD1.3 phage to lysozyme was assessed by ELISA (McCafferty *et al.*, 1990). Binding of the soluble scFvD1.3 was assessed in an ELISA by using the C-terminal Myc tag as described (Ward *et al.*, 1989). Sequencing template was prepared from phage as in Clackson *et al.* (1991) and sequenced (Sanger *et al.*, 1977) with phage T7 DNA polymerase (Sequenase, USB, Cleveland, U.S.A.).

† Change in a CDR residue.

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Table 3
Relative affinities and off-rates for D1.3 scFv fragments on phage

	Titres of scFvD1.3 phage	Titres of M1 phage	Titres of M2 phage	Titres of M3 phage
Input phage	10^9	10^9	10^9	10^9
Affinity selection with 0·2 nM HEL				
Eluted Phage	$0{\cdot}6 \times 10^6$	$1{\cdot}3 \times 10^6$	$1{\cdot}1 \times 10^6$	$2{\cdot}1 \times 10^6$
Ratio	1	2·6	2·2	4·2
Off-rate selection using 10 mM HEL to bind and then flooding				
0 min	20×10^6	24×10^6	25×10^6	27×10^6
5 min	10×10^6	19×10^6	19×10^6	25×10^6
15 min	3×10^6	11×10^6	13×10^6	21×10^6
30 min	$0{\cdot}6 \times 10^6$	5×10^6	6×10^6	12×10^6
60 min	$0{\cdot}3 \times 10^6$	2×10^6	2×10^6	5×10^6
120 min	$0{\cdot}2 \times 10^6$	$0{\cdot}5 \times 10^6$	$0{\cdot}7 \times 10^6$	1×10^6
Approx half-life of dissociation (min)	5	15	15	25

The methods are as described previously (Hawkins *et al.*, 1992). For each mutant a phage stock of standard concentration was made. The relative affinity is determined using a limiting concentration of biotinylated HEL (less than the lowest anticipated K_d) and estimating the ratio of phage eluted. For off-rate estimation the phage were saturated with biotinylated HEL and then diluted into an excess of unlabelled HEL and then captured at different times. The apparent half-life of dissociation can be estimated.

The binding affinities and kinetics of the parent scFv fragment (M0), and mutants M1, M2 and M3 were first estimated as displayed on phage. After a single round of affinity selection with 0·2 nM biotinylated lysozyme, the relative yields of phages (M0 = 1; M1 = 2·6; M2 = 2·2; M3 = 4·2) after capture were consistent with two- to fourfold improved affinities (Table 3). Likewise, after a single round of kinetic selection, the time taken for the number of bound phage to fall to half the initial number (M0 = 5 min; M1 = M2 = 15 min; M3 = 25 min), suggested a three- to fivefold slower dissociation (Table 3). After recloning into a vector for soluble expression, the mutant scFv fragments were prepared and purified on lysozyme-Sepharose. The affinities of the fragments (K_d) were measured by fluorescence quench titration, on-rates (k_{on}) by stopped-flow kinetics; off-rates were deduced from $k_{on}K_d$ (Foote & Winter, 1992). The results (Table 4) agree with the values estimated from phage.

As shown previously for an antibody of the mouse primary response (Hawkins *et al.*, 1992), and here for an antibody of the secondary response, it is possible to improve the affinity several-fold *in vitro*. However, here none of the set of mutations leading to an increased affinity (and slower off-rate) was a contact residue. Although we have not attempted to dissect the contributions of each of the mutations, three of the residues VH (Pro40 → Leu and Asn83 → Asp) and VL (Glu3 → Lys) are framework residues and relatively distant from the binding site (Fig. 2(a)). Any effect on binding of lysozyme must be indirect, for example in helping to accommodate the flexible linker polypeptide joining the VH and VK domains. The mutation VL (Thr97 → Ser) is located in VL CDR3, but also lies well outside the contact interface (Fig. 2(a)).

By contrast, the mutations VH (Asp58 → Ala) and VK (Ile29 → Thr) alter interactions with contact residues. In the structure of the complex of Fab with lysozyme (Fischmann *et al.*, 1991), VH Asp58 forms a hydrogen bond to VH Trp52, an important contact residue. VL Ile29 fits between two important contact residues VL Tyr32 and VL Trp92, and may also help to maintain the structure of L1 loop (Chothia *et al.*, 1989: and see Fig. 2(b)). Indeed,

Table 4
Dissociation constants and kinetic constants of selected mutants

Mutant	K_d (nM)	On-rate ($\times 10^{-6}$ M^{-1} s^{-1})	Off-rate (s^{-1})
Starting antibody (M0)	3·3 (0·6)	1·8	0·006
Selected by affinity (M1)	1·1 (0·2)	1·6	0·0018
Selected by off-rate (M2)	1·2 (0·3)	1·3	0·0015
Combined (M3)	0·6 (0·1)	1·6	0·00096

The affinity measurements represent the values obtained from the average of 12 sets of fluorescence quench titrations. Figures in parentheses are the standard errors. Affinity measurements were determined by fluorescence quench titrations. On-rates were determined from a series of stopped-flow measurements under pseudo first-order conditions as described and the off-rates calculated from the K_d and on-rate (Foote & Winter, 1992).

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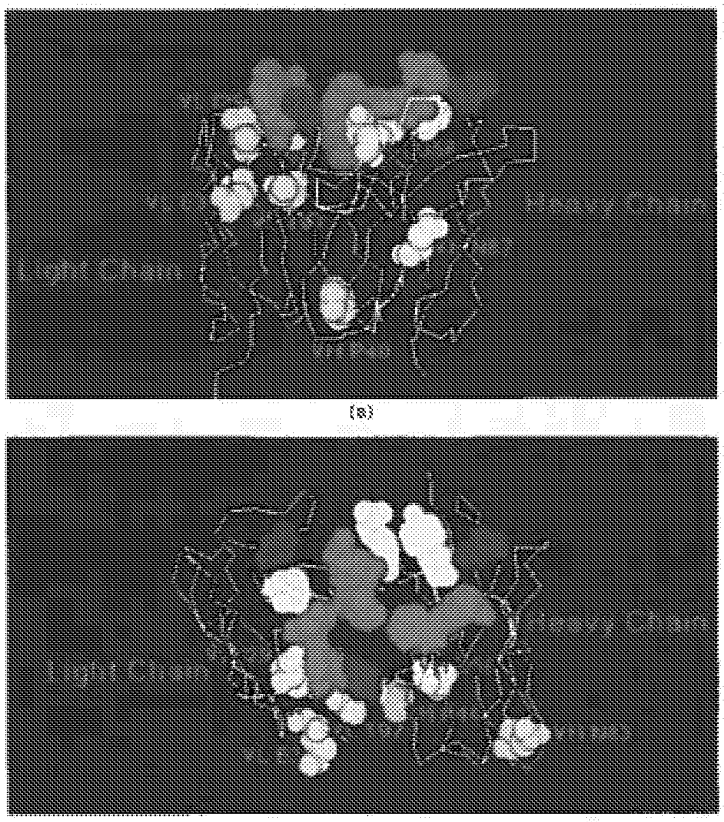


Figure 2. Two views of the binding site of the antibody D1.3 to indicate the position of the mutated residues in the higher-affinity mutant selected from a phage library. (a) The binding site seen from the side and showing how residues VH D58 and VL I29 interact with important antigen contact residues. Residue VL Thr97 although it is in VL CDR3 is not in the binding site and the others are distant from the binding site. (b) The binding site seen from above. Again the interactions of VH D58 and VL I29 with residues important for antigen binding are shown. VL I29 rests in the groove between VL Y32 (CDR1) and VL W92 (CDR3) and VH D58 makes a hydrogen bond with VH W52 across the base of the CDR2 loop.

earlier work has showed that alterations in residues that pack against the CDR loops can modulate the binding affinity for antigen (Riechmann *et al.*, 1988; Foote & Winter, 1992).

We conclude that random mutation of the D1.3 scFv fragment *in vitro* has elicited mutants with improved affinity, but that the residues contacting antigen were unchanged. The improvement in affinity must therefore be mediated by altered residues outside the antigen binding site, some of which interact with the contact residues. Such indirect interactions appear to underpin much somatic mutation *in vivo*. For example, residues of the monoclonal antibodies HyHEL-10 (anti-lysozyme) and 36-71 (anti-*p*-azophenylarsonate) arising by somatic mutation and important for the affinity of binding proved to lie outside the contact interface (Lavoie *et al.*, 1992; Sharon, 1990). However, direct interactions can play a role in affinity maturation, as with the secondary immune response to the hapten *ph*Ox in which a new contact appears to be introduced into the binding pocket for the hapten (Alzari *et al.*, 1990).

The outcome of mutating direct contacts or other residues will depend on the detailed interactions of the antigen-antibody complex. Random mutation of the contacts is likely to destroy antigen binding, but there must also be a slim chance of making greatly improved interactions. For the residues that pack against these contacts, the effects of mutation should be less extreme; indeed high affinities can be built *in vivo* in a step-wise manner (Kocks & Rajewsky, 1988; Sharon, 1990; Foote & Winter, 1992) by accumulating mutations that contribute to a slight improvement in affinity (or as above *in vitro* by combining mutants). Our work provides evidence that in the complex of the D1.3 antibody with lysozyme, the key binding contacts are provided by a band of residues at the centre of the interface, but that the affinity of binding is most

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readily improved by altering other residues. Furthermore, it shows that the binding affinity of a high-affinity antibody can be improved further by mutation and selection *in vitro*. This process may be important for clinical applications as experimental evidence suggests that higher affinity antibodies can lead to improved therapeutic effects (Schlom *et al.*, 1992).

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COVER STORY

Eculizumab's triumph over PNH

The first-of-its-kind drug has provided patients with PNH their first viable treatment option.

HemOnc Today, August 25, 2008

The FDA approval of the orphan drug eculizumab in March 2007 marked a distinct change in how physicians were able to treat patients with paroxysmal nocturnal hemoglobinuria.

The results of several clinical trials have found that eculizumab (Soliris, Alexion) not only prevented the breakdown of red blood cells but also virtually eliminated many of the quality-of-life symptoms associated with PNH, **Wendell Rosse, MD**, told HemOnc Today.

"I have been with patients with PNH for more than 50 years now," Rosse said. "It has been fascinating to observe how by scientific method we have come to understand much of the disease and the causes of the disease. Now, we finally have a rational treatment based on that understanding."

HemOnc Today spoke with Rosse, professor emeritus at Duke University and Red Cells & Hemoglobinopathies Section Editor of the HemOnc Today Editorial Board, and several other experts in the field about how eculizumab has changed PNH and the questions that are still unanswered.

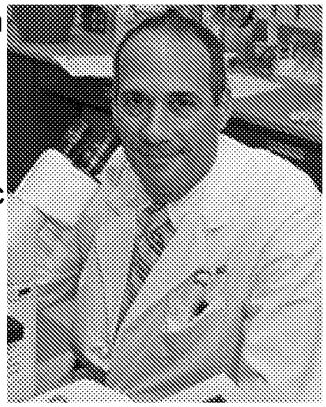
"Eculizumab has dramatically changed PNH," **Peter Hillmen, MD**, a consultant hematologist at the Leeds Teaching Hospitals NHS Trust in England, told HemOnc Today. "PNH is often diagnosed when people are in their 20s and 30s.

The symptoms of the disease — severe lethargy, abdominal pain, difficulty swallowing, hypertension and erectile problems for men — can persist for years.

"Until eculizumab arrived there was no specific treatment for PNH apart from transfusion and occasionally bone marrow transplant," he said.

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Bone marrow transplantation—which is the only thing that can cure the disease— has a 15% to 20% mortality rate, even with an identical donor, Rosse said. "It is not a very satisfactory alternative, although sometimes it is necessary," he said. "Beyond that, the only thing we had was symptomatic treatment. The hemolysis could be treated, but not very well, by high-dose prednisone, which could not be maintained on a daily dose." Iron replacement, transfusion, folic acid replacement and general medical care were all used to treat the symptoms of the disease, according to Rosse.



Robert A. Brodsky, MD, is Director, Division of Hematology, and Clara Lucas Lynn Chair in Medicine, The Johns Hopkins University.

Photo by Frederick Dubs

DISCUSS IN OUR FORUM

How do you think eculizumab has changed PNH?

"We had very little to offer patients," **Robert A. Brodsky, MD**, director of the division of hematology and Clara Lucas Lynn Chair in Medicine at The John Hopkins University, said in an interview. "I recently had a patient with PNH present in absolute crisis. Several years ago the only thing we would have had to offer him was narcotics and maybe fluids. Now that eculizumab is approved, all I have to do is call down to the pharmacy and tell them that we need to start the patient on this drug."

Prior to the drug's approval, patients with PNH had to cope with sometimes debilitating symptoms such as anemia, fatigue, thrombosis, hemoglobinuria, dyspnea, dysphagia, erectile dysfunction, hypertension and kidney problems for 10 to 15 years, with very few treatment options.

Patients with PNH whose symptoms are related to intravascular hemolysis will derive the most benefit from eculizumab, according to **Charles Parker, MD**, of the University of Utah School of Medicine, Salt Lake City. "This group of patients have a relatively large PNH clone and as a result of that have a great deal of intravascular hemolysis," he said. "Those patients, in addition to having anemia, will also have a variety of constitutional symptoms that greatly impact their quality of life."

By eliminating the intravascular hemolysis, eculizumab also ameliorates many of the symptoms of PNH.

"It relieves the symptoms of the disease in a way that many patients have not experienced since their diagnosis," Rosse said. "The esophageal spasm is gone, the abdominal pain is gone, the erectile dysfunction is gone, the hypertension is reduced and the kidney function is improved. … It makes a major difference in the way patients can live with the disease."

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Testing an orphan drug



Charles Parker

The FDA approval of eculizumab was based on the results of clinical trials that examined the efficacy of eculizumab in treating transfusion dependence in patients with PNH.

"Although the leading cause of death in PNH is thrombosis, not everyone clots, so thrombosis could not be the primary endpoint of these trials," Brodsky said. "In addition, the median survival of the disease is long, 10 or 15 years, so survival could not be an endpoint. Therefore, the researchers examined hemolysis measured through transfusion avoidance as the primary endpoint."

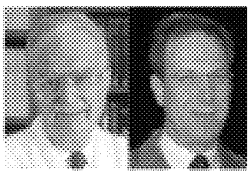
An open-label pilot study was conducted from May to December 2002 in 11 transfusion-dependent patients with PNH. Patients received weekly IV infusions of 600 mg eculizumab for four weeks, followed by a 900-mg dose, and then 900 mg eculizumab every two weeks until week 12. The results of the study indicated a decrease in intravascular hemolysis, a decrease in transfusion requirements and an improved quality of life for patients receiving eculizumab.

To test the drug under more rigorous standards, researchers from several institutions in the United States and Europe started the TRIUMPH trial. In this phase-3, double blind, randomized study, 87 patients were randomly assigned to either placebo or the pilot-study regimen of IV eculizumab for 24 weeks. Placebo was chosen instead of standard care because no method of treatment was considered an industry standard.

"The patients that went into TRIUMPH were a very restricted PNH group," Brodsky said. "They were patients who had to have a normal platelet count of over 100,000, and had to be fairly heavily red–cell-transfused dependent." Brodsky estimated that only about 10% to 20% of the patients with PNH that he sees would fit these eligibility criteria.

Results of the TRIUMPH study were published in 2006 in The New England Journal of Medicine. Forty-nine percent of the patients in the eculizumab group achieved stabilization of hemoglobin levels in the absence of transfusion, compared with none in the placebo group ($P<.001$). Eculizumab reduced intravascular hemolysis compared with placebo and there were improvements in quality of life ($P<.001$) and fatigue scores ($P<.001$) in the eculizumab group compared with the placebo group.

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Wendell Peter Rosse Hillmen

"TRIUMPH showed a dramatic effect on reduction in intravascular hemolysis," Parker said. "It was the definitive study on the efficacy of eculizumab."

Next, researchers began the SHEPHERD trial. This study was also phase-3 but was open label instead of randomized. It also relaxed its inclusion criteria to include patients with minimal transfusion requirements and lower platelet counts.

"The SHEPHERD study was a much larger, real-world population of PNH patients," Brodsky said. "This population probably captured more than 70% to 80% of patients with the disease. About 70% of patients on SHEPHERD would not have been eligible for TRIUMPH."

In this study, 97 patients were assigned to the pilot-study regimen of eculizumab for 52 weeks. Results showed an 87% reduction in hemolysis ($P<.001$). Treatment with eculizumab also resulted in an improvement in anemia and fatigue scores and a reduction in transfusion dependence.

"Both trials generated very compelling data for the use of the drug in patients with PNH whose clinical symptoms are related to intravascular hemolysis," Parker said.

Finally, all of the patients enrolled in these three studies were rolled over into an open-label extension study to examine the effect of eculizumab on thrombosis. "The major cause of morbidity and death in PNH is the tendency toward thrombosis," Rosse said. "This is because of abnormalities in the platelets. The activation of complement brings about the excessive thrombosis, particularly in the veins of the abdomen and liver."

Extension study

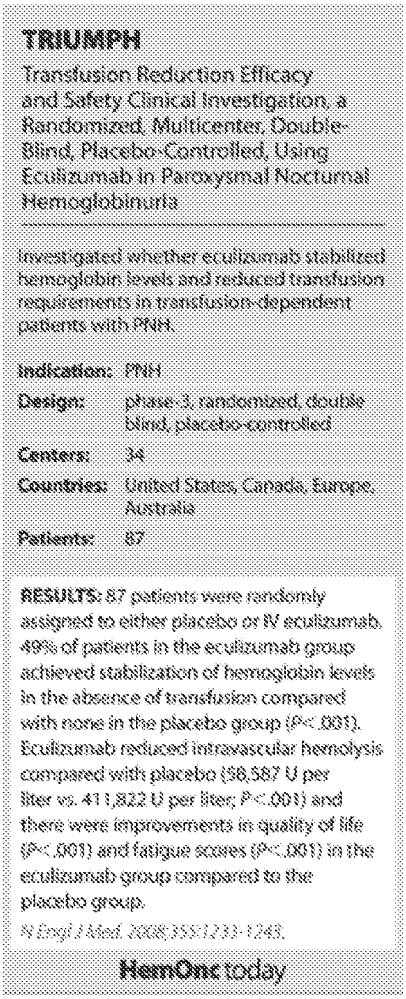
In the extension study, the researchers compared thromboembolism rate while on eculizumab with the pretreatment rate of thromboembolism in the same patients. At study end, the thromboembolism rate was 1.07 events per 100 patients years on eculizumab compared with 7.37 events per 100 patient years prior to treatment ($P<.001$). When the researchers compared an equal number of years before and during treatment, thromboembolism events were still reduced from 39 events prior to treatment to only three events on treatment ($P<.001$).

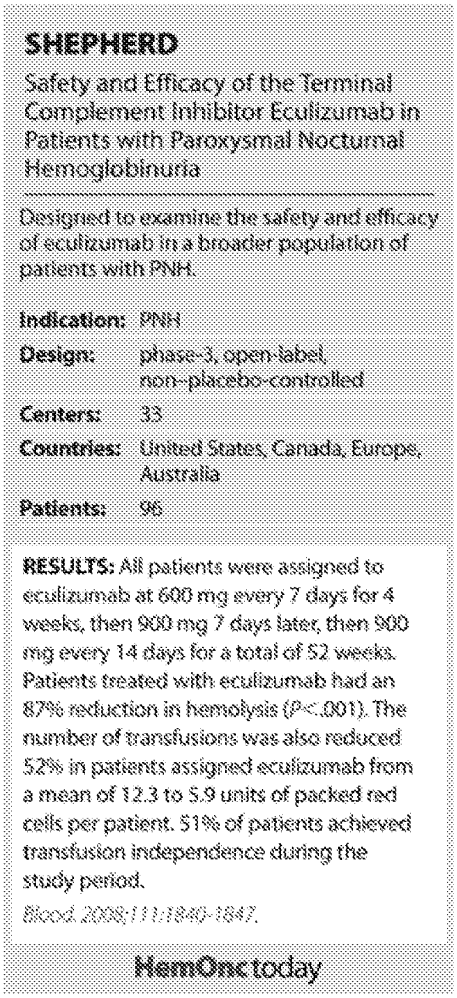
"It is very clear. There was a significant reduction in the number of thromboses in patients on eculizumab compared to patients not on the drug," Hillmen said.

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However, the results of this trial still leave some room for questions, according to Brodsky. "Only about 40% to 50% of patients with PNH develop thrombosis, or it may take a patient four to five years from diagnosis before they have their first clot," he said. "In a perfect world you would want to do a randomized trial to examine thrombosis, but the disease is so rare that it would be too expensive and take too long to do a study like that." Despite that, Brodsky said that the drug greatly reduces, if not eliminates, the risk of thrombosis.

Despite the overwhelmingly positive results of the clinical trials leading to eculizumab's approval, treatment with the drug does have some drawbacks.





Pitfalls of PNH

"One of the major pitfalls of this drug is that it is intravenous," Brodsky said. Patients being treated with eculizumab have to get IV infusions of the drug every two weeks, potentially for the rest of their lives.

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"If they stop, within a month they will have their disease back again," Brodsky said. "The IV infusion is given over 30 minutes and there is little in the way of side effects, but you still have to go to an infusion center and by the time you check in and get the IV started and the medicine comes up from the pharmacy you basically lose half a day every two weeks."

However, for patients with severe PNH, this inconvenience may be considered minor, according to Hillmen. "Prior to eculizumab, patients had to be transfused maybe once every week or two or once every few months," he said. "One of the problems with PNH is that one day you can feel reasonably OK and the next day you cannot get out of bed. It's a very difficult disease to manage, so being treated by infusion every two weeks is trivial to those patients that are badly affected."

Another drawback to the treatment is that it is extremely expensive, Rosse said. "The estimated cost is about $380,000 per year per patient." Multiply that number over 20 or 30 years and it adds up.

"The disease is so rare that the insurers are not too bothered by it," Brodsky said. "But it is still a very expensive medication." The cost limits access to treatment in developing countries.

Finally, because eculizumab is an antibody against the C5 component of complement, patients with PNH treated with the drug are at an increased risk for *Neisseria* infections, particularly *Neisseria* meningitides, Rosse said.

"Everyone is vaccinated before they start the drug," Brodsky said. "The risk of *Neisseria* meningitides on this drug is about 0.5% per year. But if you have a 20-year-old woman on it, and she will be on it for the rest of her life, over the next 60 years she probably has a 30% chance of coming down with *Neisseria* meningitides."

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FAST FACTS

Issues at Hand

1 Prior to the approval of eculizumab, patients diagnosed with paroxysmal nocturnal hemoglobinuria had few treatment options available to them and often had to deal with severe symptoms such as lethargy, erectile dysfunction and thrombosis for 10 to 15 years.

2 Eculizumab was approved in March 2007 based on the results of the TRIUMPH trial, which indicated that eculizumab helped patients achieve stabilization of hemoglobin levels, reduced intravascular hemolysis and improved quality of life.

3 Although eculizumab dramatically improves symptoms of PNH, it does not cure the disease. Physicians who are prescribing eculizumab to patients with PNH should still be aware of a patient's risk for *Neisseria* meningitides, thrombosis and extravascular hemolysis.

Questions unanswered

In addition to considering the possible pitfalls of prescribing eculizumab, there are other questions that physicians should consider when prescribing the drug.

"Eculizumab is not right for everyone with PNH and does require judgment on the part of the prescribing physician to identify the appropriate patient for treatment," Parker said.

"In patients with small PNH clones who don't have much hemoylsis, their main clinical problems are related to bone marrow failure. This is typically seen in the setting of some other bone marrow failure syndrome, particularly aplastic anemia and the refractory anemia variant of myelodysplasia," Parker said. "In those cases the focus of treatment is really on the bone marrow failure component of the disease."

In addition, a patient with a small proportion of abnormal cells probably does not need the medicine because their symptoms are not incapacitating, Rosse said.

The role of anticoagulants once a patient is started on eculizumab is also unclear, Parker added. Many patients with PNH are assigned an anticoagulant to prevent either first or recurrent thrombosis. However, according to Parker, there are little data to address whether or not to continue an anticoagulant in combination with eculizumab.

"In my opinion, anyone who has had a prior thromboembolic complication should remain on anticoagulation," Parker said. "You could probably make a better case for discontinuing anticoagulation in a patient who has never had a thromboembolic

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complication once they start eculizumab, but again that issue has not been addressed by appropriate clinical studies."

Physicians should also be aware that patients with PNH are at risk for both intravascular and extravascular hemolysis, according to Parker. "In patients who are treated with eculizumab, the intravascular hemolytic process is ameliorated almost completely by the drug, but there is another part of hemolysis ,called extravascular hemolysis, that is not blocked by eculizumab."

These patients will typically still require some transfusion and possibly other modification of their treatment to maintain an adequate hemoglobin level, according to Parker. "It is important for physicians to be aware that there are a high percentage of patients that do continue to have ongoing hemolysis, although it is almost exclusively extravascular. As we use the drug more, we are seeing more of these patients.

"One thing that is important to understand is that eculizumab does not treat the underlying clonal proliferative disease in PNH. It only treats the hemolysis by blocking complement. Ultimately, the issue is that the drug is really just treating the symptoms rather than the underlying disease. We still need to understand the pathophysiology better so that we can eventually develop an approach to cure the disease, as opposed to just ameliorating the symptoms," Parker said. – *by Leah Lawrence*

POINT / COUNTER

Should patients remain on an anticoagulant after being assigned to eculizumab?

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For more information:

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Review Article

Structure-Immunogenicity Relationships of Therapeutic Proteins

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As more recombinant human proteins become available on the market, the incidence of immunogenicity problems is rising. The antibodies formed against a therapeutic protein can result in serious clinical effects, such as loss of efficacy and neutralization of the endogenous protein with essential biological functions. Here we review the literature on the relations between the immunogenicity of the therapeutic proteins and their structural properties. The mechanisms by which protein therapeutics can induce antibodies as well as the models used to study immunogenicity are discussed. Examples of how the chemical structure (including amino acid sequence, glycosylation, and pegylation) can influence the incidence and level of antibody formation are given. Moreover, it is shown that physical degradation (especially aggregation) of the proteins as well as chemical decomposition (e.g., oxidation) may enhance the immune response. To what extent the presence of degradation products in protein formulations influences their immunogenicity still needs further investigation. Immunization of transgenic animals, tolerant for the human protein, with well-defined, artificially prepared degradation products of therapeutic proteins may shed more light on the structure-immunogenicity relationships of recombinant human proteins.

KEY WORDS: aggregation; animal models; immunogenicity; protein structure; therapeutic proteins.

INTRODUCTION

Nowadays, proteins are widely used as therapeutic agents. When the first proteins became available for therapeutic use [in the 1920s, insulin was the first therapeutic protein introduced (1)], concerns were raised about their immunogenicity. Initially, proteins from animal origin (e.g., bovine and porcine insulin) were used. The non-human origin of these proteins was believed to be the reason for their immunogenicity. Later on, proteins purified from human tissue or sera were introduced. Surprisingly, these products also proved immunogenic. These products, such as factor VIII and growth hormone, were given mainly to children with an innate deficiency. The immunogenicity was explained by their lack of immune tolerance. Also, the impurities in these products were considered to be an important reason for the immunogenicity.

The introduction of recombinant DNA techniques enabled the large-scale production of highly purified proteins identical or nearly identical to the endogenous proteins. It was hoped that this would reduce the immune responses to the proteins. Currently, more than 60 recombinant human proteins [including recombinant human(ized) monoclonal antibodies] are available for therapeutic purposes in the European Union (2). Although, in general, the level and incidence of immune responses against recombinant human proteins are low and variable, most of these products have been shown to be immunogenic. In some cases, the immunogenicity of therapeutic proteins leads to serious problems.

Immunogenicity of recombinant human proteins can have a number of clinical consequences. Binding antibodies may influence the pharmacokinetics of the proteins (1,3). High neutralizing antibody levels may result in more serious consequences, such as inhibition of the therapeutic effect or the neutralization of essential endogenous proteins. An example is the antibody formation reported after the long-term subcutaneous administration of recombinant human erythropoietin (epoetin) in patients with chronic renal failure: the antibodies also neutralize endogenous erythropoietin, which results in pure red cell aplasia (4–6).

Table I shows that several factors can influence the immunogenicity of therapeutic proteins in man. This review

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ABBREVIATIONS: CHO-rhIFNβ, recombinant human interferon beta expressed in CHO-cells; *E. coli*-rhIFNβ, recombinant human interferon beta expressed in *E. coli*; epoetin, recombinant human erythropoietin; hGH, human growth hormone; hIFNβ, interferon beta obtained from human fibroblasts; HSA, human serum albumin; humatrope, natural sequence recombinant human growth hormone; met-rhGH, recombinant human growth hormone with an extra methionine; MSA, murine serum albumin; PEG, polyethylene glycol; pit-hGH, human growth hormone purified out of human pituitaries; rhGM-CSF, recombinant human granulocyte macrophage colony stimulating factor; rhIFNα2, recombinant human interferon alpha2; rhtPA, recombinant human-tissue plasminogen activator; rmIFNα2, recombinant murine interferon alpha2; VLP, viral like particles.

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Table I. Factors Influencing the Immunogenicity of Therapeutic Proteins in Man

Factors directly affecting the immune response	Factors affecting the measured immune response
Protein structure	Timing and frequency of sampling
Immune modulatory effect of the protein	Assay methods
Formulation	Expression of titers
Contaminants and impurities	
Route, dose, frequency, and duration of administration	
Major histocompatibility complex (MHC) genotype of the patient	
Associated diseases and concomitant therapy	

Adapted from (Refs. 35 and 42).

only focuses on the formation of antibodies after administration of recombinant human proteins to man in relation to the structural properties of the protein. Monoclonal antibodies will not be included in this review, because they are at best humanized and thus not completely human (2). First, the probable mechanisms of the immune responses are discussed, followed by the factors influencing the observed antibody response. The models used for the prediction of immunogenicity will be evaluated. Next, the protein structure in relation to immunogenicity will be dealt with. In the final section, formulation aspects are considered.

MECHANISMS OF IMMUNOGENICITY

There are two basic immunological mechanisms by which therapeutic proteins induce antibodies in man. Proteins carrying foreign epitopes like the proteins from microbial origin (e.g., streptokinase) induce a classical immune reaction that involves the presentation of the epitope by antigen-presenting cells. These cells activate B-cells and T-helper cells resulting in antibody formation and in the induction of memory cells, leading to an enhanced reaction upon rechallenge.

The other mechanism by which recombinant human proteins induce antibodies is based on the breaking of immune tolerance to self-antigens (7). This tolerance is based on the elimination of the immune cells reacting with self-antigen in the thymus during early development of the individual. This central mechanism of tolerance induction only concerns self-antigens that are present in sufficient amounts in the thymus. Some B-cells may escape elimination in the thymus. Peripheral mechanisms, however, keep the B-cells directed to self-antigens under control. These B-cells may be eliminated by apoptosis when they meet their antigen. Also, receptor editing has been described as a mechanism to make these cells harmless. The most likely peripheral mechanism is the induction of functional anergy in these B-cells. Apparently, these cells are not stimulated to produce antibodies by the circulating levels of endogenous proteins such as insulin, interferon, and erythropoietin. In mice transgenic for human insulin, low levels of insulin silence the B-cells, although a few cells may escape silencing and produce antibodies. This silencing mechanism may act through antigen-antibody complexes, which react with the low affinity IgG receptor Fcγ2b on B-cells comparable with the mechanism by which antirhesus prophylaxis induces tolerance to RhD (8).

Anergic B-cells only start to produce significant amounts of antibodies after receiving a second signal or "danger" signal from T-helper cells. Bacterial endotoxins that react with Toll receptors may provide such a danger signal. This explains the production of antibodies to self-antigen associated with lipopolysaccharide (LPS). Also, oligonucleotides containing unmethylated cytosin-guanosin dinucleotides (CpG) motifs present in DNA can trigger an immune reaction to self-antigens. This T-cell–dependent activation may be weak. When self-antigens are coupled with foreign Th epitopes, only a weak IgM response is induced, unless multiple high doses of antigen are given together with immune adjuvants.

The most potent way to induce high levels of IgG independent of T-cell help is to present the self-antigen arrayed on viruses and viral-like particles (VLP). The spacing of epitopes with a distance of 5–10 nm is unique to microbial antigens, and the immune system has apparently learned to react vigorously to this type of antigen presentation. Self-antigens conjugated with papilloma VLP evoked a strong antibody response to the self-antigens (9). It is the density of the exposed self-antigens on the VLPs that determines the level of the effect. VLP pentamers had the same effect as complete particles.

Apart from the self-antigens in viral-like arrays, other mechanisms exist that lead to the formation of antibodies to self-antigens:

1. Modification of the molecules, which gives antibodies cross-reacting with the unmodified self-antigens (T-cell responses are in general specific for the modified antigens).
2. Different allotypes for the gene coding for the product. This has been shown in mice with different allotypes for IL-2. When immunized, the mice produced antibodies that reacted with all forms of IL-2. However, adjuvant was used to immunize the mice (10).
3. Binding to a non–self-antigen. This has been shown for DNA bound to T-antigen from polyomavirus (11).

PREDICTIVE MODELS FOR IMMUNOGENICITY

As we will see later, physicochemical characterization of the therapeutic proteins will not completely predict their immunogenicity.

In vivo studies on immunogenicity of recombinant human proteins in conventional animals have limited value because these proteins, in general, are foreign in animals and will induce a classical immune response. These animals only provide the opportunity to study the relative immunogenicity of different protein formulations. The best models are transgenic animals, immune tolerant for the human protein, in which breaking of immune tolerance can be studied. This has been done by Ottesen *et al.* (12), Palleroni *et al.* (13) and Stewart *et al.* (14) for recombinant human insulin, recombinant human interferon alpha2 (rhIFNα2), and recombinant human-tissue plasminogen activator (rhtPA), respectively.

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Stewart *et al.* studied the effect of the addition of an adjuvant on the immunogenicity of (mutated) rhtPA in transgenic mice (14). They immunized transgenic mice with unaltered rhtPA and with a mutant containing one amino acid substitution [rhtPA(E275)]. Mice were immunized with these proteins with or without adjuvant. The results, summarized in Table II, clearly show that the adjuvant did not increase the reaction to the 'self-protein' but increased the immune response against the altered protein in the transgenic mice. Adjuvant only stimulated the classical immune response in this model. Most of the *in vivo* immunogenicity studies of therapeutic proteins have been done with mice, either wild type or transgenic. Zwickl *et al.* (15) performed studies in rhesus monkeys. They claim that the relative immunogenicity of various forms of human growth hormone (hGH) as predicted by the monkey model was confirmed in extensive clinical trials. However, according to the authors, the monkey model might not be suitable for every protein.

The *in vivo* analysis of the immunogenicity may be complicated, if the therapeutic protein has immune modulatory effects that may interfere with antibody production (e.g., interferons). Braun *et al.* studied the immunogenicity of rhIFNα2 in transgenic mice tolerant for human IFNα2. Because of the species specificity of IFNα2, they injected recombinant murine IFNα2 (rmIFNα2) or polyIC (an interferon inducer) together with rhIFNα2 to mimic its immune modulatory effects (16). In transgenic mice, the concurrent treatment did not break the tolerance toward rhIFNα2 monomers. In wild-type mice, antibody titers to rhIFNα2 increased due to the co-injection of rmIFNα2 or polyIC (16).

PROTEIN STRUCTURE AND IMMUNOGENICITY

Factors Influencing Immunogenicity

Comparison of immune responses induced by different formulations can become complex due to the issues stated in Table I. Besides factors affecting the intrinsic response, assay variations can largely influence the measured immune response. Not only do the methods used to determine antibody levels vary, but also the ways the results are reported. For instance, in enzyme-linked immuno-sorbent assays (ELISAs) the procedure used to coat the plate with antigens is of crucial importance. Depending on the chosen procedure, antibodies directed against either native or non-native epitopes will be measured (17). In patient studies, some groups report average antibody levels but no percentages of patients with antibodies, whereas other groups only mention percentages of patients with antibodies. So, standardization and validation of the assays and data presentation (e.g., level of antibodies and number of responders) is crucial.

Protein structure is one of the factors that can affect the immune reponse in man (see Table I). Proteins are complex molecules, so that a small change at a particular site may result in a major change in the overall properties. For instance, oxidation of a few amino acids of rhIFNα2 may lead to aggregation of the molecule (18). But, in general, it is very difficult to relate a particular change in protein structure to a change in immunogenicity, as will be discussed in the sections below.

Table II. Effect of Adjuvant on Immunogenicity of rhtPA and Mutant

	Immunogen			
	rhtPA		rhtPA(E275)	
	Without adjuvant	With adjuvant	Without adjuvant	With adjuvant
Control mice	40/42	12/12	79/80	12/12
Transgenic mice	0/21	0/8	8/44	4/8

Mice were immunized with rhtPA or rhtPA(E275) (with or without Freund's adjuvant). The numbers refer to the number of mice making antibodies against the immunogen. The sera were analyzed using a standard radioimmunoprecipitation assay using polyethylene glycol to precipitate the antibodies. After Ref. 14.

Amino Acid Sequence

Divergence of the primary structure from the human counterpart explains why therapeutic proteins of animal sources are immunogenic. An example is insulin. The differences in primary structure between human, bovine, and porcine insulin are depicted in Fig. 1. Porcine insulin induced slightly higher antibody levels in patients than human insulin (1,12,19,20). Even trace amounts of bovine insulin in porcine insulin preparations increased the immune response (20). Remarkably, deletion of A19Tyr from recombinant human insulin resulted in a decrease of antibody titers to 2.2% as compared to the native form, whereas the molecule lost one third of its receptor binding activity (21). This deletion results in a small conformational change and an increase in hydrophobicity, as measured by reversed-phase high-pressure liquid chromatography (RP-HPLC). Changes in the A-chain of insulin have more effect on the immunogenicity than changes in the B-chain (12), which is not surprising as the A-chain is located at the outside of the insulin hexamers.

RhIFNα2 exists in different subtypes (Table III). RhIFNα2a and rhIFNα2c both differ in one amino acid from rhIFNα2b. The majority of the human population expresses the gene encoding rhIFNα2b (13). Differences in immunogenicity seen in patients could not be related to differences in primary structure. Antibodies from patients treated with either rhIFNα2 showed complete cross-reactivity (13).

Human growth hormone was available in three different forms: purified out of human pituitaries (pit-hGH), recombinant human growth hormone with an extra methionine (met-rhGH), and natural sequence recombinant human growth

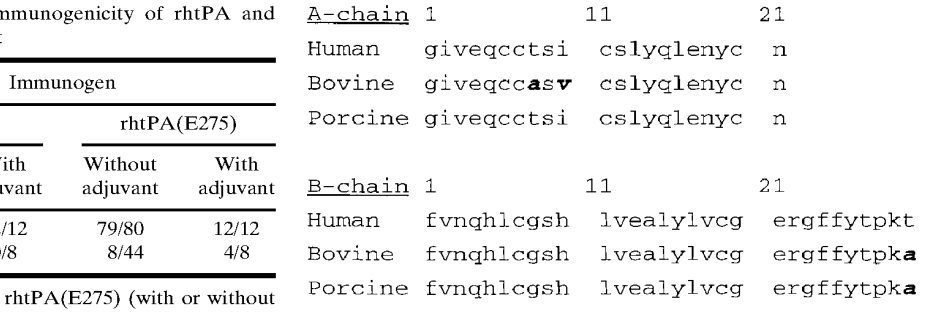


Fig. 1. The amino acid sequences of human, bovine, and porcine insulin. Differences with the human sequence are depicted in bold and italic (after Ref. 12). Disulfide bridges between A7Cys-B7Cys and A20Cys-B19Cys connect the A- and B-chain (43).

Table III. Amino Acid Differences Between Different rhIFNα Variants

Variant	AA 23	AA 34
rhIFNα2a	Lys	His
rhIFNα2b	Arg	His
rhIFNα2c	Arg	Arg

RhIFNα2a and rhIFNα2c both differ from rhIFNα2b by one amino acid (13,26).

hormone (humatrope). Pit-hGH and humatrope had the same primary structure, whereas met-hGH had one extra N-terminal methionine residue. Differences in immunogenicity were shown but could not be related to the methionine residue (15,20). More detailed studies are needed to explain the differences in immunogenicity.

RhtPA was mutated by one amino acid (R275 → E275). Transgenic mice that were tolerant for htPA were immunized with the mutant and with the nonaltered form. The altered protein elicited antibodies in a significantly higher percentage of transgenic mice than the nonaltered protein (see Table II) (14).

The impact of a mutation on the immunogenicity is dependent on the position and the type of amino acid involved. For rhtPA, a positively charged amino acid was changed into a negatively charged one. The question arises if a more subtle change (e.g., R275 → K275, both positively charged amino acids) also would give a different immune response.

As the changes in the primary structure reported are diverse and different test systems have been used (patients, monkeys, transgenic mice, and non-transgenic mice), it is very difficult to draw any general conclusions about the influences of primary structure on the immunogenicity.

Glycosylation

Several therapeutic proteins are glycosylated. Glycosylation is one of the most common posttranslational modifications. Glycosylation may differ by sequence, chain length, and position of linkage to the polypeptide chain and of branching sites (22). Glycosylation is species- and cell-specific. Moreover, the culture conditions of production cells have direct effects on N-linked glycosylation patterns (23). Thus, recombinant human glycoproteins will never have exactly the same glycosylation patterns as their endogenous counterparts.

The sugars of glycoproteins have various functions (listed in Table IV). Below we will give some examples to illustrate the effect of the glycosylation profile on the immunogenicity of glycoproteins. Other posttranslational modifications (e.g., phosphorylation, acetylation, methylation) also may influence the immunogenicity, but published data are lacking.

Four out of 13 patients who received yeast-derived recombinant human granulocyte macrophage colony stimulating factor (rhGM-CSF) formed antibodies to this product. The cross-reactivity of the antibodies with other forms of rhGM-CSF was tested (Fig. 2). Because the antibodies had the same reactivity with *Escherichia coli*–derived rhGM-CSF (no glycosylation), the authors concluded that the antibodies could not be directed against the sugars. The antibodies showed no cross-reactivity with CHO-cell–derived rhGM-CSF (O-linked and N-linked sugars). When the CHO-cell–derived rhGM-CSF was N-cleaved, cross-reactivity remained absent. However, when the CHO-cell–derived rhGM-CSF was O-cleaved, the cross-reactivity was completely regained, irrespective of the presence of N-linked sugars. It was concluded that the antibodies were directed against a part of the peptide backbone that is protected by the O-linked glycosylation in the endogenous protein. Only three patients received unglycosylated rhGM-CSF (produced in *E. coli*), and although none of them developed antibodies, definite conclusions cannot be drawn about the immunogenicity of *E. coli*–derived rhGM-CSF (24).

Table IV. Functions of Glycoprotein Glycans

Type	Function
Physicochemical	Modify solubility, electrical charge, mass, size, and viscosity in solution
	Control protein folding
	Stabilize protein conformation
	Confer thermal stability and protection against proteolysis
Biological	Regulate intracellular trafficking and localization
	Determine circulation half-life
	Modify immunological properties
	Modulate activity
	Act as cell surface receptors for lectins, antibodies, toxins, and so forth
	Participate in cell-cell interactions

After Ref. 22.

Naturally occurring human IFNα2 is O-glycosylated at Thr106 (25). No significant antigenic differences between glycosylated natural and nonglycosylated rhIFNα2 proteins have

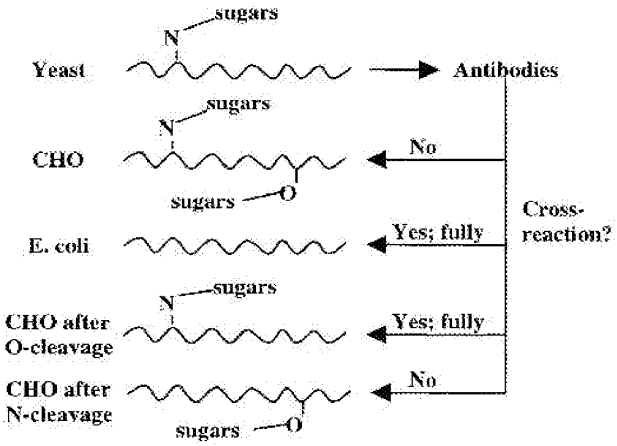


Fig. 2. Schematic representation of the cross-reactivity between antibodies against differently glycosylated forms of rhGM-CSF. RhGM-CSF produced in yeast-induced antibodies in 4 out of 13 patients. The antibodies were fully cross-reactive with rhGM-CSF produced in *E. coli*. The antibodies did not recognize the mammalian rhGM-CSF (produced in CHO-cells). When the mammalian form was O-cleaved, the antibodies did fully recognize the rhGM-CSF. This means that the antibodies are directed against a portion of the amino acid backbone protected by O-glycosylation in the native product and the product derived from CHO-cells (24).

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been identified (26). So, glycosylation probably does not affect the immunogenicity of human IFNα2.

Recombinant human interferon beta (nonglycosylated) expressed in *E. coli* (*E. coli*-rhIFNβ), and CHO-cells (CHO-rhIFNβ, glycosylated) as well as (nonrecombinant human, glycosylated) interferon beta obtained from human fibroblasts (hIFNβ) were compared for their immunogenicity. The three forms possess equal antiviral activities and a mouse monoclonal anti-hIFNβ antibody had the same affinity for the three variants. However, the antiviral activity of CHO-rhIFNβ was neutralized more effectively by mouse monoclonal anti-hIFNβ than were hIFNβ or *E. coli*-rhIFNβ, which may be explained by glycosylation differences. Furthermore, during the production process of *E. coli*-rhIFNβ, the protein is extracted from the bacteria with a strong detergent (whereas CHO-rhIFNβ and hIFNβ are secreted in the medium). This could result in a conformational change that might explain the different neutralization activity of the mouse monoclonal anti-hIFNβ (27).

In the above examples, only cross-reactivity of an antibody with glycosylation variants was tested. Clearly, sugar chains are able to mask antigenic sites *in vitro*. Whether this has implications for the immunogenicity *in vivo* remains to be seen. A complicating factor when comparing the cross-reactivity of antibodies against recombinant proteins produced in different species is that the glycans of species differ. So, when the antibodies are directed against the glycans, cross-reactivity may never be seen.

Pegylation

Chemical conjugation of proteins with polyethylene glycol (PEG, a nontoxic water-soluble polymer) reduces their immunogenicity (28). As pegylated proteins have a prolonged circulating time (29), pegylation has the potential to reduce the immune response as a result of less frequent administration schemes. In addition, it may shield immunogenic sites, like glycosylation does. In most cases, pegylation does not lead to conformational changes of the polypeptide chain (28). Important issues regarding protein activity and immunogenicity are the choice of the PEG chain length, the conjugation method, the number of PEG chains per protein molecule, and the pegylation sites (30).

Conjugates of rhIFNα2a with PEG of various molecular weights and structures (branched or linear) decreased the immunogenicity in comparison to nonpegylated rhIFNα2a. In addition, one of the pegylated forms showed no immunogenicity in mice (31,32).

The immunogenicity of monopegylated recombinant human insulin with distinct sites of substitution and varying molecular weights of the PEG was studied in mice (33). All forms, irrespective of molecular weight or site of conjugation, reduced the level of circulating insulin-specific IgG antibodies 10- to 1000-fold.

The heterogeneity in the length of the PEG chain and the site where the PEG is attached make it difficult to establish structure-immunogenicity relationships. In our opinion, the best place to attach the PEG chain would be around possible antigenic epitopes, if this does not result in decreased activity of the protein.

Physical Degradation

Proteins have complex three-dimensional structures. Physical degradation processes such as unfolding, misfolding, and aggregation of the protein can result in an immune response. Physical degradation of the protein can occur during the production and purification but can also result from improper formulation or storage or handling conditions. The choice of the formulation and the dosage form requires extensive research to guarantee the physical stability of the protein and thus minimize immunogenicity.

Especially aggregates have been shown to increase the immunogenicity of various therapeutic proteins, which might be explained by their multiple-epitope character (cf. the viral-like arrays described earlier) and/or to conformational changes of the individual aggregated protein molecules.

For instance, aggregates of insulin can lead to antibody formation (1). Evidence exists that insulin aggregates possess antigenic sites that are absent in monomers. Further research is needed to determine whether these aggregates promote the formation of antibodies against insulin monomers (34).

Aggregates were present in rhIFNα2a formulations containing human serum albumin (HSA) stored for a prolonged period at 25°C. Not only rhIFNα2-rhIFNα2 aggregates, but also HSA-rhIFNα2 aggregates were present, which might have contributed to the observed immunogenicity (13,18,35). The effect of different rhIFNα2 aggregates on the immunogenicity was studied systematically in mice. Not only rhIFNα2-rhIFNα2 aggregates isolated from an expired bulk solution were injected intraperitoneally (i.p.) in wild-type and transgenic mice tolerant for human IFNα2, but also rhIFNα2-rhIFNα2, rhIFNα-HSA, and rhIFNα2-MSA (murine serum albumin) aggregates, made with glutaraldehyde. As controls, rhIFNα2 monomers or a mixture of either HSA/rhIFNα2 or MSA/rhIFNα2 were injected. The aggregates (either one) induced antibody formation in both conventional and transgenic mice. In the transgenic mice, the rhIFNα2-rhIFNα2 aggregates and albumin (human and murine)-rhIFNα2 aggregates could break an existing tolerance toward rhIFNα2 monomers, in contrast with nonaggregated rhIFNα2 formulations with or without albumin (16).

The presence of protein aggregates in formulations of (nonrecombinant) hGH has been correlated with an increased frequency of immune responses. Three distinct patterns of antibody formation in patients were observed when different preparations of hGH were used. The patients were divided in three groups according to antibody formation (Table V). The first group of patients developed antibodies that were persistent irrespective of the length and type of hGH therapy. The second group of patients developed anti-

Table V. Role of Aggregated hGH and Patient Features in Immunogenicity of (Nonrecombinant) hGH Formulations

Group	Antibodies	Number of patients	Formulation
1	Persistent	11	50–70% aggregated
2	Transient	18	<5% aggregated
3	No antibodies	33	Different types

Patients were divided in groups according to antibody formation. See text for details. Adapted from Ref. 36. hGH, human growth hormone.

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bodies that disappeared after switching to a different preparation of hGH or when the therapy was discontinued. The third group developed no antibodies. The first group had received a preparation of hGH that contained 50–70% aggregates, the second group had received a preparation with less than 5% aggregated hGH, and the third group had received different types of hGH (either the 50–70% aggregated, less than 5% aggregated, or monomeric), as determined after the study. It was concluded that the formation of antibodies was dependent on both the level of aggregates present and patient characteristics (36).

Aggregates are a decisive factor for an antibody response. Aggregates not only increase the classical immune response in conventional animals but also break immune tolerance in transgenic animals made tolerant for the therapeutic protein.

Chemical Degradation

Oxidation and deamidation, sometimes followed by isomerization, are the major causes of chemical degradation of proteins (e.g., during storage or handling). One of the impurities found in rhIFNα2a formulations because of improper storage was an oxidized form (18). The oxidation sites were not determined. The oxidation product was isolated and compared with nonoxidized rhIFNα2a for its immunogenicity in wild-type mice. The oxidized form was more immunogenic than the nonoxidized rhIFNα2a (18,35).

Deamidation mainly occurs at asparagine residues. In this reaction, the asparagine residue is, via a succinimide intermediate, converted into an aspartate or an iso-aspartate residue (37–39). Also, glutamine residues can undergo deamidation resulting in glutamate residues. The rate of deamidation is dependent on the type of nearby amino acids and on the conformation (37,40). Not much is known about the immunogenicity of deamidated proteins. Chen *et al.* showed that antibodies can be raised specifically against the succinimide derivative of peptides, showing weak cross-reactivity with the parent peptide (41).

CONCLUDING REMARKS AND DISCUSSION

Structural changes in proteins may affect their immunogenicity. The presence of grafts (sugar or PEG chains) on the polypeptide chain may decrease their immunogenicity. There are no reports about the introduction of antigenic sites due to these additions. The most important recognized structural change known to increase the immune response is aggregation. Even small amounts of aggregates may be sufficient to elicit an immune response. The aggregation either reveals new epitopes recognized as non-self or leads to the spacing of the epitopes known to break self-tolerance. Aggregates occur in different sizes, and the proteins in the aggregates can have a native or non-native conformation. Folding probably has a great influence on immunogenicity because the misfolded protein may present different epitopes than the native protein. The effect of non-native conformers present in protein formulations on the immunogenicity is still unclear and needs to be investigated further.

To what extent do the antibodies induced by structural variants of a protein cross-react with the native protein? If the immune response is directed toward an epitope specific for the variant, the only clinical consequence may be the loss of effect of the therapeutic protein involved. However, when the immune response is directed toward an epitope present in the endogenous protein, the antibodies induced may also neutralize this endogenous protein, and serious clinical consequences may be anticipated.

The formulation of proteins plays an important role in the occurrence of immunogenicity. Excipients in the formulation added to stabilize the protein may affect its immunogenicity. The excipients could change the presentation of the protein to the immune system (i.e., in a spacing of epitopes known to be a strong stimulus for the immune system). Lyophilization of HSA-containing rhIFNα2a formulations and storage at room temperature induced aggregates (HSA-rhIFNα2a and rhIFNα2a aggregates). Removal of the HSA and storage in a refrigerator led to less aggregation and diminished immunogenicity (35).

Still, little is known about the relationship between changes in the structure of therapeutic proteins and their immunogenicity. The development of new and improvement of current analytical techniques will help in identifying impurities and non-native conformers in protein formulations. With the advent of transgenic animals tolerant for human proteins, systematic studies on the correlation between conformational and chemical changes and immunogenicity can be conducted. Well-defined degradation products can be prepared, and their immunogenicity can be compared to that of native proteins. It is unlikely that one can completely predict the immunogenicity in patients by using transgenic animal models. However, such models will at least be useful to screen formulations or structural variants for their immunogenicity and can therefore be valuable during the development of new therapeutic protein formulations as well as for the establishment of proper storage and handling conditions.

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Erythropoietin treatment during complement inhibition with eculizumab in a patient with paroxysmal nocturnal hemoglobinuria

Paroxysmal nocturnal hemoglobinuria (PNH) is characterized by intravascular hemolysis leading to anemia and other clinical manifestations. Transfusions are often required to support hemoglobin at tolerable levels. A PNH patient with aplastic anemia was treated with the complement inhibitor eculizumab, followed by concurrent treatment with recombinant human erythropoietin (rHuEpo). Eculizumab alone reduced hemolysis, increased PNH red blood cell (RBC) mass, and decreased transfusions. Addition of rHuEpo during eculizumab therapy, enhanced erythropoiesis, further increased PNH RBC mass and hemoglobin levels, and rendered the patient transfusion independent for more than two years. These data show that driving erythropoiesis during eculizumab treatment provided further benefit to a patient with PNH and underlying bone marrow failure.

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Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired genetic disorder resulting from clonal expansion of a hematopoietic stem cell containing a somatic mutation of the PIG-A gene, which results in a deficiency of glycosylphosphatidylinositol (GPI) anchored proteins from the surface of red blood cells (RBCs).[1] Absence of the GPI-anchored terminal complement inhibitor CD59 on RBCs results in an increase in complement-mediated RBC lysis leading to anemia and other clinical manifestations of the disease.[2-5] Currently, no approved therapies effectively reduce intravascular hemolysis in PNH, and patients are often dependent on transfusions to artificially support hemoglobin levels.[2]

Eculizumab is a novel humanized monoclonal antibody directed against the complement protein C5.[6] Eculizumab was previously shown to safely and effectively reduce intravascular hemolysis and transfusion requirements in patients with PNH.[7-9] Herein, we report a case study of a PNH patient with evidence of underlying aplastic anemia who was treated with eculizumab and thereafter received recombinant human erythropoietin (rHuEpo) in an effort to increase PNH RBC production during complement inhibition and thereby further reduce transfusion requirements.

Case report

A 48-year old transfusion-dependent male was diagnosed with aplastic anemia in May 1988 and with PNH in September 1993. He has been transfusion-dependent due to PNH starting in September 1993, requiring transfusions of packed red blood cells (PRBCs) every 4 to 6 weeks. He received eculizumab infusions starting May 22, 2002 and is currently dosed at 900 mg every other week. On November 6, 2002, after 6 months of receiving eculizumab, rHuEpo (NeoRecormon®) therapy was initiated at the following doses: 450 IU/kg/week in 3 divided doses during the first 2 months; 900 IU/kg/week in 3 divided doses during the next 15 months; and 750 IU/kg/week in 3 divided doses until May 3, 2006. At that time he was switched to Aranesp® at a dose of 300 mcg every 2 weeks. The dose was increased to 500 mcg every 2 weeks on 28th June 2006.

Intravascular hemolysis was assessed by measuring levels of the enzyme lactate dehydrogenase (LDH). Levels of erythropoiesis were determined by measuring reticulocyte counts. The PNH RBC count was calculated by multiplying the absolute number of RBCs by the proportion of PNH type III RBCs as assessed by flow cytometry.[10] Hemoglobin levels and PRBC transfusion requirements were also monitored. All assessments have been collected to the present date and results are reported through August 2006.

During the year prior to eculizumab therapy, the mean LDH level was 2,075 IU/L (more than 4 times that of the upper limit of the normal range), the mean hemoglobin level was 10.5 g/dL, and the mean reticulocyte count was 77.5×10^9/L (Table 1). The absolute number of PNH type III RBCs was 1.13×10^{12}/L, and the proportion of these cells constituted less than 50% of the total RBC mass. The patient required 1.8 units of PRBCs per month during the pre-treatment period (Table 1), receiving a

Table 1. Hematological parameters before and after eculizumab and rHuEpo therapies.

			Mean ± SD		
Parameter	*Upon Diagnosis of AA (May 1988)*	*Upon Diagnosis of PNH (Sept 1993)*	*Pre-treatment (1 year)*	*Eculizumab alone (0.5 year)*	*Eculizumab + RHuEPO (3.7 years)*
LDH, IU/L (normal range 150-480)	-	-	2075±1590	456 ± 76	679±146
Hemoglobin, g/dL (normal range 13.5-18.0)	7.5	9.3	10.5±1.5	10.2±0.9	11.4±1.1
Reticulocytes, $\times 10^9$/L (normal range 20-80)	-	-	77.5±10.6	96.4±29.5	205.3±43.6
White Blood Cell Count, $\times 10^9$/L	2.9	3.9	4.47±0.53	3.49±0.50	3.84±0.58
Neutrophil Count, $\times 10^9$/L	0.6	2.2	2.83±0.52	2.02±0.28	2.14±0.33
Platelet Count, $\times 10^9$/L	20	140	113.60±14.53	96.88±8.06	114.40±15.62
PNH Neutrophil Proportions (%)	-	-	99.82*	99.84±0.14	99.84±0.10
PNH Red Cell Proportions (%)	-	-	35.10±10.30	65.95±11.68	87.90±9.23
PNH type III RBCs, $\times 10^{12}$/L†	-	-	1.13±0.31	1.93±0.13	2.52±0.27
Units transfused per month	1.6	2.5	1.8	1.0	0.1

*Single reading; †Calculated as (proportion of PNH type III RBCs) x (total number of RBCs) ÷ 100.

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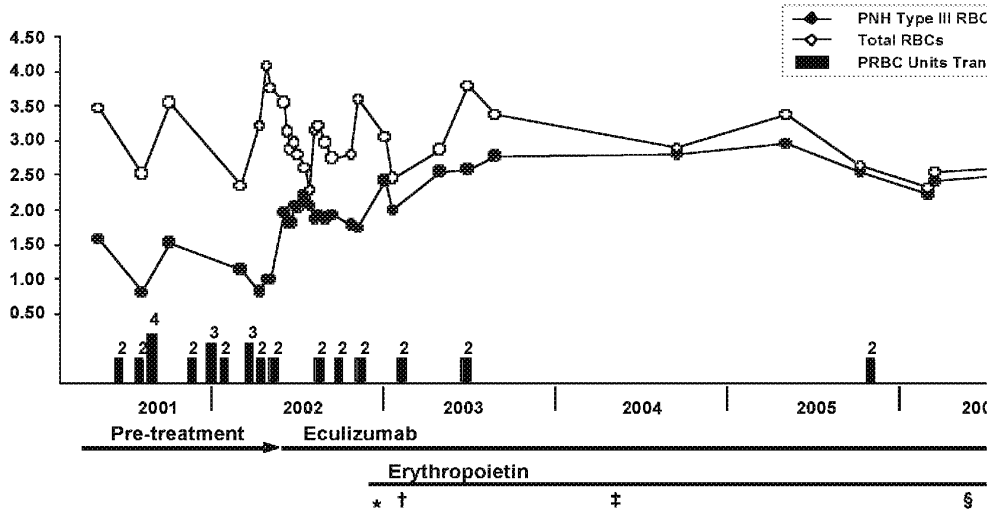


Figure 1. Effect of eculizumab and recombinant human erythropoietin on PNH type III RBC counts and transfusion requirements. The diamonds represent PNH type III RBC counts and solid bars represent the number of PRBC units transfused. The x-axis indicates time in years. Type and dose of erythropoeitin was as follows: *NeoRecormon® at 450 IU/kg/week in 3 divided doses; †NeoRecormon® at 900 IU/kg/week in 3 divided doses; ‡NeoRecormon® at 750 IU/kg/week in 3 divided doses; §Aranesp® at 300 mcg every 2 weeks; ^Aranesp® at 500 mcg every 2 weeks.

total of 9 transfusions and 22 units (Figure 1).

After starting eculizumab treatment, hemolysis was rapidly and consistently reduced as indicated by a 78% decrease in the mean LDH level (Table 1). A concomitant increase (73%) in the PNH type III RBC mass was also demonstrated, supporting enhanced survival of these cells. Further, the average number of transfusions required each month was reduced by 44%. RBC hemoglobin was stable even though transfusion requirement decreased, indicating a net increase in endogenous hemoglobin levels (Table 1).

After 6 months of eculizumab treatment, the patient received concomitant rHuEpo therapy resulting in a mean reticulocyte count increase of 113% (Table 1). This increase in erythropoiesis was associated with an additional 32% increase in the PNH type III RBC mass over that achieved with eculizumab treatment alone. In addition, RBC hemoglobin levels showed an increase from 10.2 g/dL to 11.4 g/dL during the same period. This improvement in anemia resulted in a further decrease in transfusion requirements, eventually leading to transfusion-independence for more than two years (Figure1). This transfusion coincided with a transient decrease in erythropoiesis, as evidenced by a drop in the reticulocyte count (data not shown). There was no evidence of an increase in intravascular hemolysis and LDH levels have remained within the normal range or just above the upper limit of the normal range during the entire treatment period. This patient continues to receive eculizumab and rHuEpo and has received only 1 transfusion in more than 3 years.

In patients with PNH, transfusions are often required to help maintain tolerable RBC hemoglobin. However, the level of hemoglobin in these patients is not only a function of RBC transfusions but also the underlying bone marrow dysfunction and the reduced survival of the complement-sensitive PNH RBC. Attempts to drive erythropoiesis have not generally been successful as a treatment for anemia in PNH patients. Although some patients respond vigorously to erythropoietin administration, this does not translate into a sustainable increase in the endogenous RBC mass as the PNH type III RBC is destroyed by the unopposed activation of terminal complement. In addition, increasing the PNH RBC mass in a setting where these cells are vulnerable to destruction may lead to exacerbation of the disease as intravascular hemolysis is central to the morbidities of PNH.[11]

Eculizumab, a recombinant humanized monoclonal antibody has been shown to reduce chronic intravascular hemolysis, increase PNH RBC survival, improve patient quality of life, and reduce the need for transfusion in patients with PNH.[7-9] In the recent pivotal phase 3 trial, of 43 patients on eculizumab for up to 26 weeks, the reduction in transfusions was sustained in all patients, with 51% maintaining transfusion independence.[7]

We hypothesized that reduction of intravascular hemolysis during eculizumab treatment combined with enhanced erythropoiesis should result in a net increase in PNH RBC mass and higher endogenous hemoglobin levels, thereby further reducing the need for transfusion in patients who were not rendered transfusion independent by eculizumab alone. PNH patients with clear clinical evidence of underlying bone marrow failure, and reticulocyte counts that are either low, normal or only marginally elevated, are likely the best candidates to respond to erythropoietin therapy and therefore benefit in a setting where the PNH RBC is protected. Endogenous serum erythropoietin levels may also be a valuable indicator of those more likely to respond to rHuEpo

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therapy although they were not measured in this patient.[12] However, in a meta-analysis by Littlewood *et al*, it was found that 55% of patients with erythropoietin levels >100 mU/mL still responded to rHuEpo by 8 weeks, and even 42% of patients with baseline erythropoietin levels >500 mU/mL ultimately responded;[13] although this study was performed on patients with cancer-associated anemia and may not be directly comparable to patients with AA.

Results of this case study involving a PNH patient with aplastic anemia, demonstrate that control of intravascular hemolysis with eculizumab while driving erythropoiesis further improves anemia by increasing the endogenous PNH RBC mass, thereby reducing and/or eliminating the need for transfusion support.

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Acute Leukemia

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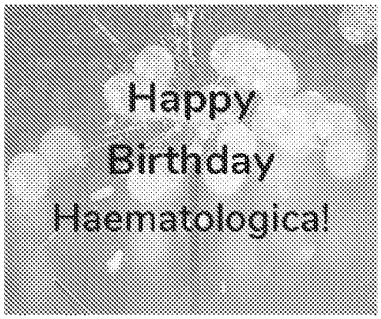
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Long-term effect of the complement inhibitor eculizumab on kidney function in patients with paroxysmal nocturnal hemoglobinuria

Peter Hillmen,[1]* Modupe Elebute,[2] Richard Kelly,[1] Alvaro Urbano-Ispizua,[3] Anita Hill,[1] Russell P. Rother,[4] Gus Khursigara,[4] Chieh-Lin Fu,[5] Mitsuhiro Omine,[6] Paul Browne,[7] and Wendell Rosse[8]

Paroxysmal nocturnal hemoglobinuria (PNH) is a debilitating and life-threatening disease in which lysis of PNH red blood cells frequently manifests with chronic hemolysis, anemia, and thrombosis. Renal damage in PNH is associated with chronic hemosiderosis and/or microvascular thrombosis. We determined the incidence of renal dysfunction or damage, defined by stages of chronic kidney disease (CKD), in a large cohort of PNH patients and evaluated the safety and efficacy of the complement inhibitor eculizumab in altering its progression. Renal dysfunction or damage was observed in 65% of the study population at baseline with 21% of patients with later stage CKD or kidney failure (glomerular filtration rate [GFR] ≤60 ml/min/1.73 m^2; Stage 3, 4, or 5). Eculizumab treatment was safe and well-tolerated in patients with renal dysfunction or damage and resulted in the likelihood of improvement as defined as categorical reduction in CKD stage ($P < 0.001$) compared with baseline and to placebo ($P = 0.04$). Improvement in renal function was more commonly seen in patients with baseline CKD Stages 1–2 (67.1% improvement, $P < 0.001$) although improvement was also observed in patients with CKD Stages 3–4 ($P = 0.05$). Improvements occurred quickly and were sustained for at least 18 months of treatment. Patients categorized at CKD Stages 3–5 did not worsen during treatment with eculizumab. Overall, 40 (21%) of 195 patients who demonstrated renal dysfunction or damage at baseline were no longer classified as such after 18 months of treatment. Administration of eculizumab to patients with renal dysfunction or damage was well tolerated and was usually associated with clinical improvement. Am. J. Hematol. 85:553–559, 2010. © 2010 Wiley-Liss, Inc.

Introduction

Intravascular hemolysis and the presence of cell-free plasma hemoglobin cause many of the serious clinical sequelae associated with various hemolytic disorders [1]. Paroxysmal nocturnal hemoglobinuria (PNH) is a rare and life-threatening blood disease that is clinically defined by significant intravascular hemolysis [2,3]. PNH arises from an acquired clonal genetic deficiency of glycosylphosphatidylinositol-linked proteins on the surface of blood cells [3,4]. In particular, PNH red blood cells lack complement regulatory proteins, which render them susceptible to hemolysis mediated by the terminal components of complement. The release of excessive red cell hemoglobin during intravascular hemolysis usually exceeds the capacity of the hemoglobin scavenging molecule, haptoglobin, leading to high levels of cell-free hemoglobin, and subsequently clinical manifestations of PNH [1].

Five-year mortality in patients with PNH is approximately 35%, and median survival is approximately 10–15 years [2,5]. Kidney failure contributes to 8%–18% of PNH-related deaths [6]. Virtually all patients with PNH have evidence of kidney damage by biopsy or imaging techniques or at postmortem examination [7–16]. Previous reports showed that repetitive exposure to cell-free hemoglobin causes renal hemosiderin accumulation, tubulointerstitial inflammation, and kidney damage [17].

Cell-free plasma hemoglobin in patients with PNH has also been shown to deplete nitric oxide (NO), leading to smooth muscle dystonias and pulmonary hypertension [18,19]. NO is also a critical molecule in the regulation of vascular tone with the greatest effect on the afferent renal arterials [20]. Alterations in renal blood flow because of the sequestration of NO can have a direct effect on the glomerular filtration rate (GFR) and renal plasma flow [20–23].

Eculizumab (Soliris®) is a humanized monoclonal antibody that targets complement protein C5, thereby preventing production of the potent proinflammatory mediator C5a and the assembly of the terminal complement complex (also called the membrane attack complex) during complement activation [24]. In clinical studies, treatment of PNH patients with eculizumab resulted in a reduction in hemolysis, cell-free plasma hemoglobin, and NO depletion [19,25]. Data from treated patients also demonstrated an improvement in anemia, fatigue and quality of life, and a reduction in thrombotic events [25–28]. Herewith, we report the incidence of renal dysfunction or damage and evaluate the effect of long-term eculizumab treatment on the progression of this serious and life-threatening morbidity in a large, multinational cohort of patients with PNH enrolled in a phase 3b open-label extension study.

Hillmen has received honoraria and research funding from and served as a consultant to and member of the Advisory Board of Alexion Pharmaceuticals. Kelly has received grant support from and served on Advisory Boards for Alexion. Omine received research funding and is a consultant for Alexion. Urbano-Ispizua has served on the Alexion Advisory Board. Rother and Khursigara are employees of Alexion Pharmaceuticals and own stock in the company.

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Conflict of interest: Nothing to report

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Methods

Study design and patients. Kidney function and the incidence of clinical kidney damage event rates were assessed in patients from three independent parent clinical studies (the Phase 2 pilot study [26,29] and its extensions, the Phase 3 TRIUMPH study, [25] and the Phase 3 SHEPHERD study [28]) and the subsequent common Phase 3 extension study [30]. All protocols were approved by the institutional review board at each center, and all patients gave written informed consent.

The open-label, 12-week, Phase 2 pilot study was conducted in 11 patients at two study centers in the United Kingdom [26]. Patients who completed the pilot study were eligible to enroll in a 1-year extension study [29] followed by a 2-year extension study. Patients 18 years or older who had received at least four red cell transfusions in the previous 12 months and had a PNH Type III erythrocyte population ≥10% were eligible to enroll in the pilot study. Patients who were taking stable doses of immunosuppressive drugs (e.g., cyclosporine), warfarin, and iron supplements were permitted to continue them during the study. Eculizumab was dosed at 600 mg via intravenous infusion every 7 days for 4 doses, 900 mg 7 days later, and 900 mg every 14 days as a maintenance dose.

The double-blind, placebo controlled, 26-week, Phase 3 efficacy and safety study (TRIUMPH) was conducted in 87 patients in the United States, Europe, Australia, and Canada [25]. Patients 18 years or older, with at least four red cell transfusions in the previous 12 months, a PNH Type III erythrocyte population ≥10%, platelets ≥100 × 10^9/l, and lactate dehydrogenase (LDH) ≥1.5 times the upper limit of normal were eligible to enroll. Exclusion criteria were described previously [25]. Patients who were taking stable doses of immunosuppressive drugs, anticoagulants, and iron supplements were permitted to continue during the study. Patients were centrally randomized (1:1) and stratified (according to red cell units transfused in the past year) to receive either placebo or eculizumab. Study medication was dosed as follows: eculizumab (600 mg) or placebo via intravenous infusion every 7 days for 4 doses; eculizumab (900 mg) or placebo via intravenous infusion 7 days later; followed by a maintenance dose of eculizumab (900 mg) or placebo via intravenous infusion every 14 ± 2 days for a total of 26 weeks of treatment.

The open-label, 52-week, Phase 3 study (SHEPHERD) was conducted in 97 patients in the United States, Europe, Australia, and Canada [28]. Inclusion criteria for patients were broader than those for patients enrolled in TRIUMPH, as the pretreatment transfusion eligibility was adjusted to one or more red cell transfusions in the previous 24 months and the minimum platelet count requirement was lowered to 30 × 10^9/l; all patients received the identical eculizumab dose as in the TRIUMPH study.

After completion of the parent clinical studies, 187 patients enrolled in the common open-label, 102-week, Phase 3 extension study (195 patients were enrolled in parent studies) [30]. Placebo-treated TRIUMPH patients entering the Phase 3 extension study received the same eculizumab regimen outlined for the TRIUMPH and SHEPHERD studies. Eculizumab-treated patients from the pilot, TRIUMPH, or SHEPHERD studies who entered the Phase 3 extension study continued to receive the 900 mg maintenance dose of eculizumab. Doses of immunosuppressive drugs, anticoagulants, and iron supplements could be altered at the discretion of the treating physician.

Outcome measures. For each of the 195 PNH patients entered into the eculizumab clinical trials, evidence of renal dysfunction or damage, as measured by chronic kidney disease (CKD) stage, was evaluated at baseline, 6, 12, and 18 months. All patients were required to have a urinalysis and serum creatinine measures at baseline and during the trial. Patients with a positive dipstick urine protein test were considered to have evidence of proteinuria. The GFR was estimated using the modification of diet in renal disease study formula equation, as defined previously [31,32]. CKD stages were defined using the Kidney Disease Outcomes Quality Initiative as: Stage 5 CKD, GFR less than 15 ml/min/1.73 m^2; Stage 4 CKD, GFR 15–30 ml/min/1.73 m^2; Stage 3 CKD, GFR 30–60 ml/min/1.73 m^2; Stage 2 CKD, GFR 60–90 ml/min/1.73 m^2 and evidence of kidney damage, which may include spot urinalysis with proteinuria or by abnormal imaging findings; and Stage 1 CKD, GFR greater than 90 ml/min/1.73 m^2 and evidence of kidney damage, which may include spot urinalysis with proteinuria or by abnormal imaging findings. Patients were classified as not having renal dysfunction or damage if they failed to meet the criteria for Stages 1–5 CKD [33]. Initial measurements were performed at screening before study enrollment. An improvement in renal function was defined as a categorical documented reduction in CKD stage level (e.g., a change from Stage 4 to Stage 3 or Stage 3 to Stage 1) or fulfilling the criteria of no CKD. A worsening in renal function was defined as a categorical documented increase in CKD stage level (e.g., a change from Stage 3 to Stage 4 or Stage 1 to Stage 2). No change in renal status was defined as the patient remaining in the same CKD stage or the continued absence of CKD. In addition, major clinical kidney (MCK) events were determined by querying all medical history events before the use of eculizumab and all adverse events during treatment with eculizumab. Medical history events before diagnosis of PNH were excluded. By definition, all medical history events obtained in these analyses were nonfatal. MCK events were defined as event terms with severe reversible or irreversible renal damage including acute or chronic renal failure, renal insufficiency, renal impairment, dialysis, and procedures (i.e., hemodialysis and/or placement of catheters for dialysis, renal artery angioplasty, and ureteral catheterization), nephrotoxicity, scarred or necrotic kidney, pyelonephritis, glomuleropathy, or Stages 3–5 CKD (complete list described in Table V). MCK events during placebo treatment in the TRIUMPH study were considered with the group of events before eculizumab treatment. These analyses included all events during the period commencing from the first eculizumab dose in the parent studies until the last follow-up of either the last dose of eculizumab or the data base lock in July 2007.

TABLE I. Baseline Demographics of Patients in All PNH Studies (N = 195)

Description	
Gender, male	89 (45.6)
History of AA or MDS	59 (30.3)
History of previous thrombosis	62 (31.8)
Use of cyclosporine	13 (6.6)
Use of NSAID	17 (8.7)
Age (yr)	39 (18–85)
Disease duration (yr)	5.5 (0.12–38.5)
PNH type III RBC proportions (%)	32.3 (2.4–98.8)
Reticulocyte counts (×10^{12}/l)	0.164 (0.036–0.757)
Lactate dehydrogenase level (U/l)	2139 (499–10,300)
Platelet count (×10^9/l)[a]	149 (23–547)

Data are represented as n (%) or median (range).
AA, aplastic anemia; MDS, myelodysplastic syndrome; RBC, red blood cell.
[a] N = 194.

Statistical analysis. The changes for all patients in each CKD stage were compared within each stage and across the overall treatment population with the observations at screening using chi-square analyses and the null hypothesis that the probability of worsening CKD stage was equal to the probability of improving CKD stage. The changes in CKD stage for patients treated with eculizumab in the TRIUMPH study and for patients treated with placebo in the TRIUMPH study were also each compared with the respective observations at screening using chi-square analyses and the null hypothesis that the probability of worsening CKD stage was equal to the probability of improving CKD stage for each treatment group. The changes in CKD stage for patients treated with eculizumab in the TRIUMPH study were compared with the changes in CKD stage for patients treated with placebo in the TRIUMPH study using chi-square analyses and the null hypothesis that the probability of worsening and improving with eculizumab treatment was equal to the probabilities with placebo treatment. The incidence rate of MCK events was tabulated, and the MCK event rate per patient-year was determined for the preeculizumab and the eculizumab treatment periods for the overall patient population. A time-to-event analysis was performed for MCK events before trial enrollment. The MCK event rate was determined as events per 100 patient-years before and during eculizumab treatment. Patients with a MCK event before treatment were included in the patients evaluated to have a potential MCK event during eculizumab treatment. Event rates were compared on an intention-to-treat basis with the Wilcoxon signed-rank test.

Results

Patient characteristics

Baseline characteristics of patients in each of the parent clinical studies are shown in Table I. Ninety-six percent of all eligible patients chose to enroll in the common extension study.

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TABLE II. Renal Characteristics in Patients

CKD stage	Baseline, n (%)	18-month treatment, n (%)
Stage 5	3 (1.5)[a]	1 (0.6)
Stage 4	7 (3.6)	8 (4.7)
Stage 3	30 (15.4)	25 (14.8)
Stage 2	48 (24.6)	18 (10.7)
Stage 1	36 (18.5)	14 (8.3)
No CKD	69 (35.4)	103 (60.9)

[a] One of the three patients was receiving dialysis at treatment entry.

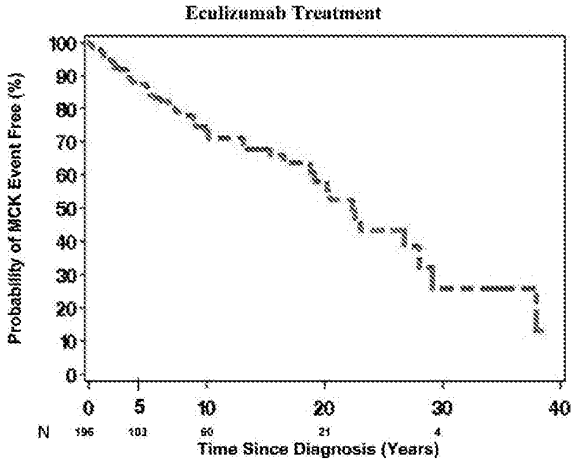


Figure 1. Time to major clinical kidney event in 195 patients with PNH prior to eculizumab treatment. The Kaplan-Meier probability of patients progressing to an MCK event. Event free; 85% (95% CI 77.9–89.6) at 5 years, 73% (95% CI 63–80) at 10 years, 56% (95% CI 42–67) at 20 years, and 25% (95% CI 10–44) at 30 years. CI, confidence interval. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Renal dysfunction or damage was a common finding in patients with PNH as 65% of the study population exhibited Stages 1–5 CKD (Table II). Approximately 21% of patients had renal insufficiency comprising CKD Stages 3, 4, or kidney failure (Stage 5). Age can play a contributing role into the development of CKD. The National Kidney Foundation (NKF) estimates the prevalence of CKD Stages 3–5 in people 65 years or older is 17%. Of the 15 patients 65 years or older who entered the trials, we found that 10 (66%) patients had CKD stages 3–5. Of the 180 patients younger than 65 years, we observed 114 (63%) had CKD and 30 (16.7%) had CKD stages 3–5 (four times higher then the general population with CKD stages 3–5) [32]. Taken together, these data suggest that patients with PNH are equally likely to have CKD at different ages, although CKD at an older age may be more likely to be more severe. In addition, there were 14 patients with a history of acute renal failure, 5 of which had required dialysis, which showed at least partial recovery and 21% (3/14) of these patients subsequently developed chronic renal insufficiency (chronic GFR < 60 ml/min/1.73 m^2).

MCK events were common in the PNH patient population, as 27% of patients (52 of 195) were diagnosed with an event, 67% of which occurred within 10 years of diagnosis. Further, the Kaplan-Meier probability of patients progressing to an MCK event was 15 of 103 (15%) at 5 years, 16 of 60 (27%) at 10 years, 9 of 21 (44%) at 20 years, and 3 of 4 (75%) at 30 years as indicated in Figure 1.

Effect of eculizumab on renal function

Eculizumab administration was associated with a reduction in median LDH from 1,832 to 292 U/l ($P < 0.0001$) at 6 months in patients with more severe renal damage (CKD Stages 3–5) and sustained out to 18 months (274 U/l; $P < 0.001$). In similar fashion, eculizumab reduced LDH levels from 2,722 to 372 U/l at 6 months in patients with CKD stages 1–2 ($P < 0.001$) and was sustained out to 18 months (318 U/l: $P < 0.001$).

TABLE III. Effect of 6-Month Eculizumab Treatment on Renal Function in the Placebo-Controlled TRIUMPH Study

	Change in stage of CKD with treatment			
Baseline stage	Improvement (%)	No change (%)	Worsening (%)	P[a]
Eculizumab				
All patients (n = 41)	29.3	65.9	4.9	0.005
Stages 3–5 (n = 9)	11.1	88.9	0.0	0.30
Stages 1–2 (n = 17)	64.7	23.5	11.8	0.006
Placebo				
All patients (n = 42)	16.7	69.0	14.3	0.78
Stages 3–5 (n = 9)	22.2	66.7	11.1	0.56
Stages 1–2 (n = 14)	35.7	50.0	14.3	0.25
Eculizumab vs. placebo				0.04[b]

[a] The comparison (except for eculizumab vs. placebo) performed for each group or for all patients, tested the null hypothesis that patients are as likely to improve as they are to worsen.

[b] The comparison of eculizumab vs. placebo tests the null hypothesis that the probability of patients improving or worsening with eculizumab is equal to the same probabilities with placebo.

In the randomized, placebo-controlled, double-blind TRIUMPH study, eculizumab treatment was associated with a significant improvement in renal function, demonstrated by improvement in CKD stages, compared with placebo treatment ($P = 0.04$; Table III). Left untreated, on balance patients were not likely to improve (16.7% improve/14.3% worsen: $P = 0.78$). In contrast, PNH patients treated with eculizumab were 6.0 times more likely to improve than worsen in renal function, showing a significant improvement at 6 months compared with baseline (29.3% of patients improved and 4.9% of patients worsened; $P = 0.005$). Patients with CKD Stages 1–2 at baseline were six times more likely to improve than worsen (Table III; $P = 0.006$) with eculizumab treatment, whereas kidney function did not change with placebo ($P = 0.25$). The improvement in kidney function with eculizumab treatment in patients with CKD Stages 3–5 at baseline in the TRIUMPH study was not significant (limited patient number, n = 9).

As indicated in Table IV, long-term eculizumab treatment significantly increased the likelihood of improved renal function in all CKD stages ($P < 0.001$). The improvements occurred early and were sustained to at least 18 months of treatment. When combining data from all three clinical trials, eculizumab-treated patients were four times more likely to improve than worsen at 6 months (31.7% improve/7.9% worsen), and this outcome improved to six times more likely to improve over 18 months of treatment (Table IV). Patients with CKD Stages 3–5 before eculizumab treatment were four times more likely to improve than worsen (22.9% improve/5.7% worsen; $P = 0.05$) with subsequent eculizumab treatment at 18 months and patients with milder renal dysfunction (Stage 1 or 2) at baseline were 25 times (67.1% improve/2.7% worsen, $P < 0.001$) more likely to improve than worsen with eculizumab treatment.

CKD Stages 3–5 is characterized by estimated GFR (eGFR; <60 ml/min/1.73 m^2). The mean eGFR across the CKD Stages 3–5 cohort demonstrated a trend in improvement at 18 months of eculizumab treatment but did not reach statistical significance. However, the mean change in eGFR in patients treated with eculizumab who improved with CKD Stages 3–5 at baseline was clinically significant: 10.3 ml/min/1.73 m^2 at 6 months, 12.8 ml/min/1.73 m^2 at

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TABLE IV. Effect of Long-Term Eculizumab Treatment on Renal Function

Baseline stage	Change in stage of CKD with treatment			
	Improvement[b]	No change[b]	Worsening[b]	P^a
6 months				
All patients (n = 189)	31.7%	60.3%	7.9%	<0.001
Stages 3–5 (n = 40) (mean change in GFR, ml/min/1.73 m^2)	20.0% (+10.3)	75.0% (−0.3)	5.0% (−2.9)	0.05
Stages 1–2 (n = 81)	64.2%	24.7%	11.1%	<0.001
12 months				
All patients (n = 179)	35.2%	58.1%	6.7%	<0.001
Stages 3–5 (n = 39) (mean change in GFR, ml/min/1.73 m^2)	20.5% (12.8)	76.9% (−0.6)	2.6% (−4.2)	0.02
Stages 1–2 (n = 77)	71.4%	23.4%	5.2%	<0.001
18 months				
All patients (n = 166)	34.3%	60.2%	5.4%	<0.001
Stages 3–5 (n = 35) (mean change in GFR, ml/min/1.73 m^2)	22.9% (11.5)	71.4% (0.3)	5.7% (−6.3)	0.05
Stages 1–2 (n = 73)	67.1%	30.1%	2.7%	<0.001

[a] The comparison is performed for each group or for all patients at each time point, testing the null hypothesis that patients are as likely to improve as they are to worsen.

[b] Improvement, no change, and worsening as defined in the methods section.

TABLE V. Major Clinical Kidney Events Before and During Eculizumab Treatment

All patients, N = 195	Pretreatment	Eculizumab treatment
MCK event rate (events per 100 patient-years)	4.22	2.10
Patient years	1683.6	381.6
MCK events (n)	71	8
CKD stages 3–5[a]	22	
Renal impairment	12	5
Acute renal failure	7	
Pyelonephritis	5	
Renal failure	3	2
Hemodialysis	3	
Renal dialysis	2	
Kidney stone	2	
Other[b]	15	1
Eculizumab vs placebo		$P < 0.001$[c]

[a] Patients with CKD but no documented history of renal events.

[b] Includes bilateral atherosclerotic renal artery stenosis, calculus of kidney, hematuria, hemochromatosis, kidney abscess, nephrotic syndrome, mild secondary hydronephrosis, nephrotoxicity, proliferative glomerulonephritis, renal colic, scarred kidney, hypertension, insertion of left ureteric stent, and potassium wasting nephropathy.

[c] P value based on signed rank test.

12 months, and 11.5 ml/min/1.73 m^2 at 18 months of treatment (Table IV). Of the 46 patients with CKD Stage 2 at baseline (defined as GFR between 60 and 90 ml/min/1.73 m^2 and evidence of proteinuria), 63% improved at 6 months of treatment (vs. 13.0% deteriorated), and this improvement was sustained for 18 months (68% improved vs. 2.3% worsened). Of the 36 patients with CKD Stage 1 at baseline, 65%, 71%, and 66% showed improvement in CKD after 6, 12, and 18 months of treatment, respectively. Ninety-four percent of patients with CKD Stage 1 at baseline maintained eGFR of ≥90 ml/min/1.73 m^2 and with complete elimination of proteinuria after 18 months of treatment.

Overall, during the course of 18 months of eculizumab treatment, 40 of 195 (21%) patients classified with renal dysfunction or disease were no longer classified as having CKD. This included 5 patients initially with predialysis Stage 3, 20 patients with Stage 2, and 15 patients with Stage 1. Of the five patients with CKD Stage 3 at baseline, the median change in eGFR with eculizumab treatment was 14.6 ml/min/1.73 m^2, and all patients with proteinuria had a resolution of their proteinuria. Twenty patients with baseline CKD Stage 2 no longer had CKD (CKD Stage 0) after 18 months of eculizumab treatment. Objective evidence of renal damage, as indicated by proteinuria, was eliminated with eculizumab treatment in these patients, and 15%, 17%, and 25% of patients achieved normal eGFR (≥90 ml/min/1.73 m^2) at 6, 12, and 18 months of treatment, respectively.

In patients with either one or no transfusions in the 12 months before study entry (n = 22), the distribution of renal dysfunction or disease by stage of CKD at baseline was similar to the overall population (Stage 4, 4.5%; Stage 3, 13.6%; Stage 2, 22.7%; Stage 1, 18.2%; No CKD, 40.9%). After 6 months of treatment with eculizumab, patients with minimal or no transfusion support exhibited either an improvement in renal dysfunction stage (27%) or a stabilized renal function (73%), and no patients worsened ($P = 0.008$).

Eculizumab treatment improved CKD irrespective of patient age. An improvement with eculizumab treatment was similar in patient groups younger and older than 65 years at 18 months (no significant difference in eculizumab benefit between ≥65 and <65 years; $P = 0.10$). The likelihood of an improvement in CKD with eculizumab treatment at 18 months stratified by age quartile was statistically significant in all age quartiles (Q1 < 29 years, $P < 0.001$; Q2, 29–39 years, $P = 0.009$; Q3, 39–51 years, $P = 0.004$; Q4, >51 years, $P = 0.009$). Patients with CKD Stages 1–2 at baseline showed a significant improvement in CKD with eculizumab treatment at all age quartiles. Although there was a significant improvement in CKD in the overall study population with baseline CKD Stages 3–5, when analyzed by the smaller cohorts of age quartiles, there was a demonstrated trend in improvement but it did not reach statistical significance in the individual age quartiles (n = 0, n = 6, n = 7, and n = 22 patients with Stages 3–5 CKD at baseline in each of the age quartiles, respectively).

The effect of eculizumab treatment on clinically identified MCK events is shown in Table V. The MCK event rate was reduced 50% from 4.22 events per 100 patient-years before eculizumab treatment to 2.10 events per 100 patient-years with eculizumab treatment ($P < 0.001$).

Safety

Eculizumab was safe and well tolerated. Safety observations are consistent in all three parent studies and the open-label extension study. Most treatment-emergent Adverse Events (AEs) (95.0%) were mild or moderate in severity, and 90.8% of AEs were determined to be unrelated to study drug. The most frequently occurring treatment-emergent AEs were as follows: nasopharyngitis (39.6%), headache (36.9%), and upper respiratory tract infection (31.0%). The most frequently reported AE related to study drug by preferred term was headache (15.5%). Notably, headaches were generally mild and treated with over-the-counter analgesics. As reported previously, the frequency of headaches declined after the first two treatment doses and were similar in frequency to that observed with placebo. The most common Serious Adverse Events (SAEs) reported were associated with infections and infestations (12.8% of patients), nervous system disorders (4.3%), and blood and lymphatic disorders (8.0%). The majority of SAEs were moderate in intensity and unrelated to the study drug. Two patients experienced meningococcal infections during the study. Both patients fully recovered. One patient discontinued treatment, and one patient continued treatment with study drug. Positive tests for human anti-human antibodies (HAHA) were rare, recorded in only five patients, transient, not sustained in any patient, and not associated with any pharmacokinetic or clinical effect. Three deaths were reported during this study. Two deaths

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were determined to be unrelated to study drug, and in one patient, drug discontinuation could not be excluded as a contributing factor

Discussion

Because of the recognized high incidence (65%) of renal dysfunction or damage at baseline in patients from the TRIUMPH study [27] and the serious and life-threatening nature of poor renal function in patients with PNH [12], analyses were performed to assess the overall effect of eculizumab treatment on renal function. Eculizumab treatment was significantly more likely to improve CKD stage than placebo treatment through 6 months. Long-term eculizumab treatment resulted in a significant improvement and prevention of worsening of renal dysfunction or damage at all stages of baseline disease. Patients were six times more likely to improve renal function at 18 months of eculizumab treatment, rather than deteriorate, with 34% of patients showing improvement in renal function.

CKD stage was selected as the primary measure of renal outcome, as opposed to solely eGFR, to align our results with clinically validated assessments of renal function in other populations, as recommended by the NKF [33]. The assessment of CKD Stages 1–2 identifies the importance of early damage, as measured by proteinuria, because eGFR can remain at a relatively high functional level ($\geq$60 ml/min/1.73 m^2), but the presence of proteinuria indicates further objective evidence of clinically significant glomerular and/or tubular damage. Further, NKF guidelines recommend treatment of the comorbid conditions in patients with CKD Stages 1–2 that may contribute to the increase in renal damage or proteinuria to ultimately slow progression of renal dysfunction [33]. In alignment with these NKF guidelines, the results of this clinical study indicate that blocking the on-going complement-mediated hemolysis with eculizumab treatment was associated with a significant improvement in CKD and also resulted in a reduction in the percent of patients who progressed (from 11.1% at 6 months to 2.7% at 18 months of eculizumab treatment). The staging of CKD Stages 3–5 is defined by eGFR $<$ 60 ml/min/1.73 m^2, a measure of poor renal filtration, and without the concomitant requirement for proteinuria [33]. Therefore, for patients with baseline Stages 3–5 CKD, in alignment with NKF guidelines, the current analysis focused on categorical changes in eGFR, with particular focus on maintenance of renal function and delay of deterioration. In this study, results demonstrate that 22.9% of patients with CKD Stages 3–5 improved (vs. 5.7% worsened) and 71.4% maintain stable renal function. There was a mean increase of 11.5 ml/min/1.73 m^2 eGFR in the patients with baseline CKD Stages 3–5 who improved with eculizumab treatment.

The observation that a substantial proportion of patients treated with eculizumab stabilized renal function during 18 months of treatment is clinically important. In the general population, patients without PNH with CKD Stages 1–5 followed for an average of 4.2 years, the rate of CKD stage progression has been reported as 12 patients per 100 patient-years [34]. During 1.5 years of treatment with eculizumab, the rate of CKD stage progression is 2.5 patients per 100 patient-years. Further, the rate of improvement of CKD in the general population was reported as 0.9 patients per 100 patient-years over 4.2 years [34]. In contrast, treatment with eculizumab is associated with an improvement rate of 35.2 patients per 100 patient-years. In PNH patients with more severe CKD Stages 3–5 at baseline, the rates of CKD improvement during treatment with eculizumab is 15.3 patients per 100 patient-years. The observed improvements with eculizumab treatment demonstrate the significant contribution of chronic terminal complement-mediated hemolysis to the progression of CKD in patients with PNH.

Clark et al. [12] previously reported that 32% (6/19) of patients with PNH had creatinine clearance of $<$60 ml/min/1.73 m^2 (CKD Stages 3–5), indicating kidney damage. The exact mechanisms responsible for kidney damage in patients with PNH are unclear, but a substantial number of investigations implicate chronic hemolysis leading to hemosiderin accumulation, inflammation, and renal scarring. Magnetic resonance imaging (MRI) studies reproducibly show that virtually all patients with PNH are observed to have renal cortical areas of low signal intensity including patients with PNH who have lower levels of hemolysis indicated by no hemoglobinuria for more than 8 years, aplastic anemia, myelodysplastic syndrome (MDS) or chronic myelomonocytic leukemia (CMML), or with smaller PNH clones [7,10,16,35]. Cortical abnormalities detected by intravenous pyelogram have been noted in patients with lower levels of hemolysis as represented by the absence of overt hemoglobinuria [12]. Autopsy or biopsy findings typically show heavy hemosiderin accumulation in the proximal tubules, hemoglobin tubular casts, signs of chronic interstitial nephritis, and fibrosis [12]. When examined, functional tubular defects are commonly found with impaired ability to concentrate urine, renal tubular acidosis, decreased reabsorption of phosphate, glucosuria with normal blood sugar, and aminoaciduria [12,36].

The renal cortical damage in PNH is typical of intravascular hemolysis and not generally found in patients with extravascular hemolysis [11]. Cell-free plasma hemoglobin can lead to progressive tubulointerstitial inflammation with upregulation of monocyte chemoattractant protein-1 and nuclear factor-κB and upregulation of both interstitial and basement membrane extracellular matrix components. In addition, hemosiderosis in the kidney with direct generation of free radicals has also been suggested as a mechanism for both acute and chronic kidney inflammation and fibrosis in PNH [14,37]. Hemosiderosis was found in 9 of 10 patients in a recent MRI study of patients with PNH [16]. The fate of iron deposits in the kidneys of these patients after resolution of hemolysis with eculizumab treatment is currently under investigation. Terminal complement inhibition with eculizumab would be expected to inhibit the direct hemolysis-mediated renal damage and the damage secondarily because of inflammation associated with kidney injury.

Eculizumab administration is associated with an immediate and sustained reduction in intravascular hemolysis in all treated patients [25,26,28]. Inhibition of terminal complement with eculizumab rapidly and consistently reduced intravascular hemolysis to near normal levels in patients from all PNH clinical studies, whereas placebo treatment in the TRIUMPH study showed no effect [25]. Moreover, the reduction in intravascular hemolysis with eculizumab treatment resulted in similar reductions in cell-free plasma hemoglobin to near normal levels [19]. In a previous study, treatment with eculizumab significantly reduced the load of cell-free plasma hemoglobin delivered to the kidneys in patients with PNH, as indicated by the 96% ($P < 0.001$) reduction in the frequency of hemoglobinuria [26].

Biochemical analyses of patients with PNH have demonstrated high levels of cell-free plasma hemoglobin and the resultant depletion of NO [1,19]. Interestingly, reduction of available NO in animal models results in an increase in renal arteriole resistance, a reduction in renal plasma flow and GFR, and subsequently poor renal function [20–23]. In addition, pooled data from 310 human subjects with normal renal function demonstrated that administration of the NO synthase inhibitor L-NG-monomethyl arginine significantly reduced renal plasma flow and that GFR was dependent

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on NO synthase activity, suggesting a direct role of NO in glomerular hemodynamics [20]. In clinical trials, eculizumab rapidly reduced hemolysis and cell-free plasma hemoglobin, resulting in a repletion of NO in patients with PNH [19]. Although eculizumab treatment improved renal function at all stages of CKD, it was particularly noteworthy in patients classified with Stage 1 or 2 CKD at baseline. In a higher proportion of patients with Stage 1 or 2, eculizumab treatment resulted in improvement in renal function (progression to less severe CKD stage or transition to no CKD) in 67% of treated patients. In addition, the improvements in renal function in all stages of CKD occurred rapidly (within 6 months). Taken together, these data suggest the possibility that the availability of NO and subsequent changes in renal vascular tone after eculizumab treatment may play a role in the improvement of renal dysfunction or damage demonstrated in this study. Nonetheless, whether renal dysfunction is because of NO-mediated vasomotor responses in the renal arteries or renal disease because of the direct effects of cell-free plasma hemoglobin and hemosiderin, mitigation of hemolysis in these patients with eculizumab improves renal function. The impact of eculizumab on retarding progression and/or improving function may be more substantial with intervention early in the course of the disease.

It has been suggested that large vessel thrombosis or microvascular thrombosis may contribute to renal damage in patients with PNH [12]. However, clinical observations have been only infrequently supportive of large vessel thrombosis as a cause of renal dysfunction or damage in PNH with many studies demonstrating renal damage in the absence of large vessel occlusions [9,13–15]. In addition, no incidence of renal vein or renal arterial thrombosis was reported among 195 patients in this study population [30]. Nonetheless, subclinical microvascular venous or arterial thrombosis could conceivably contribute to renal insufficiency in PNH [12].

The study population presented here indicates that the overall prevalence of renal dysfunction or disease in PNH, as measured by CKD staging, is increased 6.6-fold over that observed in the general population in the United States [32]. It is possible that drug therapies occasionally used in PNH patients such as cyclosporine or non-steroidal anti-inflammatory drugs (NSAIDs) may contribute to renal dysfunction or damage. However, in our study, 6.6% of patients were taking cyclosporine and 8.7% were on NSAIDs, making it unlikely that these concomitant medications significantly impacted the analyses.

Later-stage renal dysfunction or failure is a major cause of morbidity and mortality in PNH [6,12]. In this study, of the 40 patients with later stage CKD (Stages 3–5), 10 patients had been identified as specifically having chronic renal impairment in a review of their medical histories. Eighteen of these 40 patients had been identified to have had one or more MCK event, and 22 had not been diagnosed with any renal disorder. These data suggest that kidney damage has been underappreciated as a significant symptom or clinical consequence of PNH.

Eculizumab was safe and well tolerated, consistent with the safety findings in all three parent clinical trials. In keeping with established precautions, all patients were vaccinated with a meningococcal vaccine at least 14 days before initial eculizumab treatment.

In summary, PNH is a life-threatening blood disorder in which patients are at risk for end-organ damage and organ failure because of chronic hemolysis. Renal dysfunction or damage is a common and progressive medical complication in patients with PNH that also contributes to mortality in these patients. The current findings show that long-term treatment with eculizumab in patients with hemolytic PNH requiring transfusions substantially improved kidney function or reduced progression of kidney disease as a consequence of controlling the intravascular hemolysis.

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bjh research paper

Long-term safety and efficacy of sustained eculizumab treatment in patients with paroxysmal nocturnal haemoglobinuria

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Summary

Paroxysmal nocturnal haemoglobinuria (PNH) is characterized by chronic, uncontrolled complement activation resulting in elevated intravascular haemolysis and morbidities, including fatigue, dyspnoea, abdominal pain, pulmonary hypertension, thrombotic events (TEs) and chronic kidney disease (CKD). The long-term safety and efficacy of eculizumab, a humanized monoclonal antibody that inhibits terminal complement activation, was investigated in 195 patients over 66 months. Four patient deaths were reported, all unrelated to treatment, resulting in a 3-year survival estimate of 97·6%. All patients showed a reduction in lactate dehydrogenase levels, which was sustained over the course of treatment (median reduction of 86·9% at 36 months), reflecting inhibition of chronic haemolysis. TEs decreased by 81·8%, with 96·4% of patients remaining free of TEs. Patients also showed a time-dependent improvement in renal function: 93·1% of patients exhibited improvement or stabilization in CKD score at 36 months. Transfusion independence increased by 90·0% from baseline, with the number of red blood cell units transfused decreasing by 54·7%. Eculizumab was well tolerated, with no evidence of cumulative toxicity and a decreasing occurrence of adverse events over time. Eculizumab has a substantial impact on the symptoms and complications of PNH and results a significant improvement in patient survival.

Keywords: eculizumab, paroxysmal nocturnal haemoglobinuria, phase III, long-term therapy, haemolysis.

Paroxysmal nocturnal haemoglobinuria (PNH) is a rare, progressive and life-threatening haematopoietic stem cell disorder. It is characterized by complement-mediated intravascular haemolysis and a prothrombotic state (Brodsky, 2009; Hillmen *et al*, 2010). PNH may be diagnosed in patients of all ages, but the median age is in patients in their early 30s (Socié *et al*, 1996; Nishimura *et al*, 2004). Despite best historical supportive care, including transfusions and

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anticoagulation management, PNH is fatal in approximately 35% of patients within 5 years of diagnosis (Hillmen *et al*, 1995; Socié *et al*, 1996; Kelly *et al*, 2011).

The disease arises from a somatic mutation in the phosphatidylinositol glycan class A gene (*PIGA*), which disrupts synthesis of glycosylphosphatidylinositol (GPI), a molecule that anchors proteins to cell membranes (Takeda *et al*, 1993). The mutation results in the reduction or complete absence of the GPI-anchored complement regulatory proteins CD55 and CD59, which renders the cells sensitive to complement-mediated destruction (Rother *et al*, 2005; Kelly *et al*, 2009). All blood cells are susceptible to complement-mediated attack, although red blood cells are the most susceptible to lysis (Parker, 1991; Pu & Brodsky, 2011).

Complement activation in PNH is manifested as chronic haemolysis that leads to the release of free haemoglobin and the subsequent depletion of nitric oxide. Consumption of nitric oxide leads to vaso-occlusion and platelet activation and results in the common morbidities seen in PNH, such as fatigue, dyspnoea, recurrent abdominal pain, dysphagia, chest pain and pulmonary hypertension. More importantly, chronic haemolysis renders PNH patients at a greater risk of thrombotic events (TEs), renal insufficiency and other organ damage, and premature mortality. Compared with the general population, patients with PNH have a 62-fold higher risk of a TE (McKeage, 2011) and a six-fold greater risk of chronic kidney disease (CKD) (Hillmen *et al*, 2010). TEs have been reported to account for up to 67% of PNH-related deaths (Hillmen *et al*, 2007), while CKD has been reported to account for 8–18% of disease-related mortality (Clark *et al*, 1981; Nishimura *et al*, 2004).

Historically, supportive care measures used in PNH, such as transfusions, anticoagulation or long-term use of steroids and pain medication, leave patients with a poor quality of life and are associated with adverse events. In addition, supportive care measures do not prevent the occurrence of catastrophic events, such as thromboses and CKD, that have recently been associated with elevated risk of premature mortality (Hillmen *et al*, 2004, 2006, 2007, 2010; Rother *et al*, 2005; Brodsky *et al*, 2008; de Latour *et al*, 2009; Hill *et al*, 2010; Kelly *et al*, 2011).

Eculizumab, a humanized monoclonal antibody, inhibits the terminal complement cascade by binding uniquely to human complement protein C5, thereby inhibiting the formation of pro-inflammatory, prothrombotic C5a, and C5b, with subsequent inhibition of assembly of the membrane attack complex (Hillmen *et al*, 2006; Brodsky *et al*, 2008). Although eculizumab suppresses the terminal complement system, the proximal complement system, responsible for opsonization of microorganisms and clearance of immune complexes, remains intact and fully functional (Rother *et al*, 2007). A series of multinational clinical trials demonstrated that eculizumab therapy is well tolerated, leads to a rapid and clinically significant reduction in intravascular haemolysis and, thus, provides substantial clinical benefits (Hillmen *et al*, 2004, 2006, 2007, 2010; Rother *et al*, 2005; Brodsky *et al*, 2008; Hill *et al*, 2010). By inhibiting terminal complement activation and intravascular haemolysis, treatment with eculizumab significantly reduces TEs (Hillmen *et al*, 2007), transfusion requirements (Hillmen *et al*, 2006) and pulmonary hypertension (Hill *et al*, 2010, 2012) and significantly improves renal function (Hillmen *et al*, 2010), gastrointestinal pain (Hill *et al*, 2005a) and patient quality of life (Hillmen *et al*, 2006).

Clinical benefit occurs rapidly in patients with PNH following the initiation of eculizumab. Here we report on continuous administration of eculizumab (for up to 66 months) and the impact of long-term therapy on clinical outcomes in PNH. In addition, we provide greater insight into the safety profile of long-term eculizumab therapy for PNH.

Methods

Study design and patients

This manuscript reports on the long-term safety and efficacy of eculizumab in 195 patients with haemolytic PNH who had participated in one of three prospective parent trials: the Phase II pilot study (Hillmen *et al*, 2004; Hill *et al*, 2005b) and its extensions, the Phase III TRIUMPH (Transfusion Reduction Efficacy and Safety Clinical Investigation, a Randomized, Multicenter, Double-Blind, Placebo-Controlled, Using Eculizumab in Paroxysmal Nocturnal Haemoglobinuria) study (NCT00122330) (Hillmen *et al*, 2006), or the Phase III SHEPHERD (Safety in Hemolytic PNH Patients Treated With Eculizumab: A Multi-Center Open-Label Research Design) study (NCT00130000) (Hillmen *et al*, 2007; Brodsky *et al*, 2008). At the end of these initial studies, 187 of the 195 patients (95·9%) enrolled in an open-label extension study (Fig 1). All patients had a minimum of 10% PNH red blood cells at enrolment in the parent trials and were vaccinated with a meningococcal vaccine at least 14 d before the first eculizumab infusion in the parent studies. It was recommended that all patients be revaccinated at intervals of between 2·5 and 3 years, with meningococcal vaccine titre assays collected prior to and within 1–2 months of the revaccination. All three parent trials employed the same dosing regimen: 600-mg infusions of eculizumab every week for 4 weeks, followed 1 week later by a single 900-mg dose, and then a maintenance dose of 900 mg every 14 (±2) days until the end of the study. In the extension study patients continued to receive the maintenance dose of eculizumab.

The first clinical investigation of eculizumab was an open-label, 12-week, Phase II pilot study that enrolled 11 patients at two UK study centres, all of whom continued into two extension studies: the first of 52 weeks duration and the second of 104 weeks duration. The positive outcomes from this study (Hillmen *et al*, 2004, 2007) resulted in the initiation of two pivotal phase III studies.

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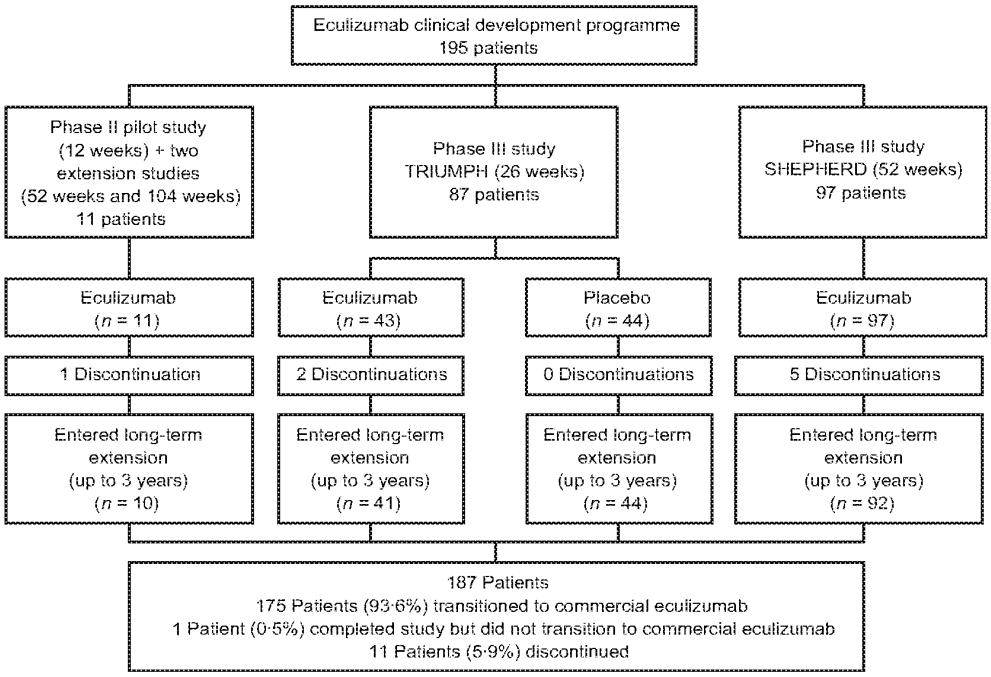


Fig 1. Phase II and III PNH clinical trials overview.

The first pivotal study, TRIUMPH, was a 26-week, double-blind placebo-controlled trial that included 87 patients randomized to either eculizumab (n = 43) or placebo (n = 44) (Hillmen *et al*, 2006). Patients were required to have had at least four transfusions during the previous 6 months for anaemia or anaemia-related symptoms, platelet counts $\geq 100 \times 10^9$/l and lactate dehydrogenase (LDH) levels $\geq$ 1·5-fold the upper limit of the normal range.

The open-label, single-arm SHEPHERD trial lasted 52 weeks and included 97 patients. The patient eligibility criteria for this study were broadened to include patients with fewer transfusion requirements (as low as one transfusion within 24 months prior to initiating eculizumab treatment) and more severe bone marrow dysfunction (platelet counts as low as 30×10^9/l) (Hillmen *et al*, 2007; Brodsky *et al*, 2008).

The long-term extension study comprised a 104-week treatment period and a 16-week post-treatment follow-up period for any patients who terminated treatment early. Patients who completed the 2-year long-term extension before eculizumab was commercially available or accessible were allowed to continue receiving the investigational product until the drug was approved in their country.

Study endpoints

Efficacy assessments were performed at least every 2 weeks from the time of initiation of eculizumab therapy in the parent study. Efficacy measures included patient survival, degree of haemolysis (as measured by blood LDH levels), TE incidence rate, mean change from baseline in haemoglobin and the number of units of transfused packed red blood cells (PRBCs) administered. Assessments of renal function were performed over the duration of the study by determining the CKD stage using formulas for estimated glomerular filtration rate (GFR) developed by the Modification of Diet in Renal Disease Study Group (Levey *et al*, 1999; Stevens *et al*, 2006). Specifically, stage 0 was defined as GFR >90 ml/min/1·73 m^2 with no evidence of kidney damage; stage 1 as GFR >90 ml/min/1·73 m^2 with evidence of kidney damage; stage 2 as GFR 60–90 ml/min/1·73 m^2 with evidence of kidney damage (proteinurea assessed by dipstick or abnormal imaging findings); stage 3 as GFR 30–60 ml/min/1·73 m^2; stage 4 as GFR 15–30 ml/min/1·73 m^2; and stage 5 as GFR <15 ml/min/1·73 m^2. The number of transfusion-independent, compared with transfusion-dependent, patients was assessed over time. The baseline period was defined as the 6 months prior to the initiation of eculizumab. At treatment evaluation, transfusion-independent patients were defined as those who did not require a blood transfusion during the previous 6 months, and transfusion-dependent patients had received at least one blood transfusion in the previous 6 months.

Safety assessments included solicited monitoring of adverse events (AEs), clinical laboratory tests and vital signs. AEs were coded using the Medical Dictionary for Regulatory Activities (MEDDRA®) version 6.1, with AEs relating to infection and infection-related serious AEs (SAEs) tabulated separately consistent

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with the expanded MedDRA preferred terms (http://www.meddramsso.com). In addition, all AEs were reviewed for events that could potentially be related to an infection and classified accordingly. AEs were assessed for severity and classified as mild (events requiring minimal or no treatment and not interfering with the patient's daily activities), moderate (events resulting in a low level of inconvenience or concern with the therapeutic measures and possibly causing some interference with functioning) or severe (events that are usually incapacitating and interrupting usual daily activities that may require systemic drug therapy or other treatment).

An SAE was defined as any event that resulted in death, was immediately life threatening, required inpatient hospitalization or prolongation of existing hospitalization, resulted in persistent or significant disability/incapacity, or was a congenital anomaly/birth defect. Important medical events that did not result in death or require hospitalization and were not life threatening may have been considered SAEs if, based upon appropriate medical judgment, they required medical or surgical intervention to prevent one of the outcomes listed previously.

Statistical analyses

Baseline was defined as the pre-eculizumab value collected from the parent study, except for those patients in the TRIUMPH study who received placebo where baseline was the value collected at the final visit of this study. Least-squares mean changes from baseline in LDH levels were analysed using a repeated-measure analysis of variance model, including time of treatment, parent study and parent study treatment as fixed effects. The signed-rank test was used to analyse mean changes from baseline in haemoglobin. Change in CKD stage was analysed using the Mantel–Haenszel chi-squared test. An improvement in renal function was defined as a categorical documented reduction in CKD stage, and a worsening in renal function was defined as a categorical documented increase in CKD stage.

Comparisons of the reporting of AEs by patients during the first 26 weeks of treatment with eculizumab and the last 26 weeks of treatment were tested using a one-tailed exact McNemar test using matched-pairs data. For any patient who remained in the study for <52 weeks, the incidence of AEs reported during the first 26 weeks of treatment was compared with the incidence of AEs from 26 weeks + 1 d until the patient's last dose of eculizumab.

Two patient populations were identified for statistical analyses. All patients who had completed at least 26 weeks of randomized treatment in one of the parent studies and had received at least one dose of eculizumab in the extension study were included in the safety population; patients from this population who had no major protocol violations were included in the per-protocol population. All analyses were performed on an intent-to-treat basis. A P value of <0.05 was considered to be statistically significant.

Table I. Baseline patient characteristics.

Parameter	Eculizumab (n = 195)
Age, years	
Median (range)	39·7 (18·3–85·0)
Mean (SD)	41·3 (14·37)
Median disease duration (range), months	81·5 (12·0–483·8)
Gender, n (%)	
Female	106 (54·4)
Male	89 (45·6)
Race, n (%)	
Caucasian	176 (90·2)
Black	7 (3·6)
Asian	6 (3·1)
Other	6 (3·1)
History of aplastic anaemia, n (%)	56 (28·7)
History of myelodysplastic syndrome, n (%)	3 (1·5)
Haemoglobin, g/l	
Mean (SE)	93·7 (1·14)
Range	48·0–143·0
Lactate dehydrogenase, u/l (upper limit of normal: 223 U/l)	
Mean (SE)	2293·3 (84·38)
Median	2129
Range	499–10 300

SD, standard deviation; SE, standard error.

Results

Study population and baseline characteristics

Baseline characteristics of all 195 patients at the start of the parent trials are shown in Table I. Overall median age was 39·7 years (range: 18–85 years); more than 90% of the patients were Caucasian and 54% were female. The majority of patients (92%) were <65 years of age. Twenty-nine percent of patients had a history of aplastic anaemia and 1·5% had a history of myelodysplastic syndrome.

The entire period of eculizumab administration across the parent and extension trials was 66 months, although a 36-month cut-off was used for safety and efficacy assessments to ensure that there were a sufficient number of patients for robust statistical analysis. Of the patients included in this 36-month cut-off, the overall median treatment duration was 30·3 months, with a range of 10·0–66·1 months (interquartile range: 26·2–33·1 months). During maintenance dosing, 21 patients (10·8%) deviated from the protocol dosing interval by receiving treatment <12 d apart on at least one occasion. Of the original 195 patients, 19 patients (9·7%) discontinued treatment over a period of 66 months: nine discontinued because of an AE (see 'Safety'), seven withdrew consent, two were discontinued based on the decision of the investigator and one was

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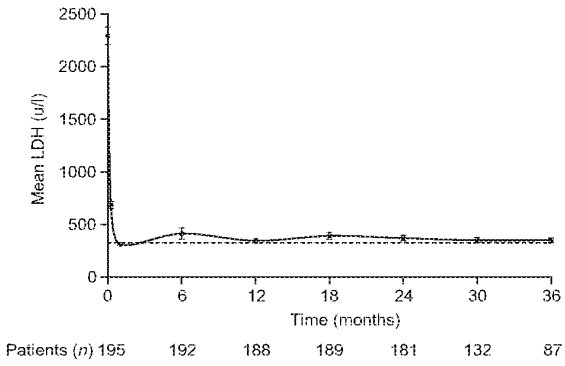


Fig 2. Lactate dehydrogenase level, a marker of intravascular haemolysis, was rapidly and consistently reduced from baseline ($P < 0{\cdot}001$ at all time points from 1 month) following initiation of eculizumab therapy. Dashed line represents the upper limit of the normal range (103–223 u/l). The decrease in number of patients was due to the transition of patients to commercially available eculizumab. LDH, lactate dehydrogenase.

noncompliant with the protocol (Fig 1). Eight of the discontinuations (six withdrawn consents and two AEs) occurred during a parent trial, and the remaining 11 discontinuations (seven AEs, two investigator's decision, one withdrawn consent, one non-compliance) occurred during the long-term extension study.

Intravascular haemolysis

All patients showed a rapid decrease from baseline in serum LDH, a marker for intravascular haemolysis, which was generally sustained with continued treatment, although throughout the course of the study some patients did have transient increases in LDH. Prior to the first administration of eculizumab, median LDH concentration was 2129 u/l (range: 499–10 300 u/l; upper limit of normal: 223 u/l). One month after eculizumab treatment initiation, median LDH decreased to 263 u/l (range: 93–1208 u/l; $P < 0{\cdot}0001$; Fig 2), a relative reduction of 87·6%. This decrease was maintained with sustained eculizumab treatment; the median LDH value at 36 months was 279 u/l (range: 88–1417 u/l), a relative reduction from baseline of 86·9%.

Plasma levels of eculizumab and haemolytic activity were reviewed for 140 patients: 43 patients from the TRIUMPH study and 97 patients from the SHEPHERD trial. Following the first dose of eculizumab, trough concentrations <35 μg/ml were observed in 49 of 135 patients (36·3%) with available samples. Overall, 36 of these 49 patients (73·5%) exhibited haemolysis as measured by a validated pharmacodynamic assay (haemolysis >20%). Multiple occurrences of trough eculizumab concentrations <35 μg/ml were observed in 14 of 140 reviewed patients (10·0%). These patients generally had more rapid eculizumab clearance (>0·4 ml/h/kg) or shorter eculizumab half-life (<130 h) than the respective median values of the trial populations as a whole.

None of the patients with breakthrough haemolysis discontinued treatment with eculizumab. In seven of the 14 patients (50·0%), the breakthrough haemolysis was successfully mitigated, as evidenced by reductions in LDH concentrations, by decreasing the eculizumab dosing interval to <14 d. None of these patients experienced SAEs relating to haemolysis. In the whole population, 21 of 195 patients (10·8%) had a median of 22 decreases (range: 3–124) in dosing intervals.

Haemoglobin levels and transfusion requirements

Treatment with eculizumab resulted in a steady increase in the serum haemoglobin concentration from baseline (93·7 g/l), including a mean increase from baseline of 5·5 g/l (range: −32·0 to 55·0 g/l) to 99·2 g/l after 2 weeks of treatment and 10·4 g/l (range: −42·0 to 81·0 g/l) to 104·7 g/l by 24 months, which was maintained at 36 months (Table II). The percentage of patients who achieved transfusion independence increased with sustained treatment (Fig 3). The percentage of patients achieving transfusion independence was 82·1% (64 of 78) by the last 6 months of treatment, compared with only 8·2% (16 of 195) in the 6 months prior to the start of treatment, a relative increase of 90.0%. Fourteen of 78 patients (17·9%) continued to require transfusions between months 30 and 36. Despite the need for transfusions, these 14 patients maintained a significant reduction in LDH from baseline levels ($P < 0{\cdot}0001$), and the number of units of PRBCs transfused over the course of the study significantly decreased from a mean of 11·2 units in the 6 months prior to starting eculizumab to 3·5 units between months 30 and 36 ($P = 0{\cdot}0001$). The change from baseline in the number of units of PRBCs administered to all patients receiving transfusions over the course of the study is presented in Table II.

Thrombotic events

Prior to initiation of eculizumab, 63 of 195 patients (32·3%) experienced 124 TEs, both venous and arterial, over a total of 1683 patient-years. These TEs included deep-vein thrombosis, hepatic vein (Budd-Chiari syndrome), cerebrovascular accident, mesenteric thrombosis and pulmonary embolism. The percentage of patients free from TEs increased from 67·7% before treatment to 96·4% during treatment: seven patients (3·6%) reported a total of 10 TEs over 467·1 patient-years. Of these seven patients, five had a history of TEs prior to starting treatment with eculizumab and four were receiving concomitant anticoagulation therapy (including warfarin and low-molecular-weight heparins). Of the 10 TEs reported during eculizumab treatment, five were reported as thrombophlebitis/deep vein thrombosis (three events occurred in the same patient), one as deep vein thrombosis, one as retinal vein thrombosis and one as a possible thrombosis in a fistula. The remaining patient experienced portal vein thrombosis and splenic infarcts. The

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Table II. Mean number of packed red blood cells transfused and haemoglobin concentrations throughout the study.

	Units of packed red blood cell transfused				Haemoglobin (g/l)*			
Study period, months	*n*	Mean (SE) [range]	Mean (SE) [range] change from baseline	*P* value	*n*	Mean (SE) [range]	Mean (SE) change from baseline	*P* value
Baseline	164	5·3 (0·22) [1–13]			195	93·7 (1·14) [48–143]		
0–3†	74	4·4 (0·33) [1–13]	−1·5 (0·38) [−8 to 6]	0·0001	195	99·2 (1·05) [64–136]	5·5 (0·96)† [−32 to 55]	<0·0001
3–6	76	4·7 (0·37) [1–19]	−1·5 (0·42) [−9 to 9]	0·0007	193	101·6 (1·15) [65–146]	7·7 (1·41) [−34 to 56]	<0·0001
6–9	61	4·8 (0·40) [2–14]	−1·1 (0·50) [−11 to 9]	0·0293				
9–12	58	4·6 (0·41) [2–18]	−1·7 (0·54) [1–13]	0·0025	190	100·5 (1·23) [36–152]	6·8 (1·51) [−54 to 55]	<0·0001
12–15	62	3·6 (0·29) [1–12]	−2·5 (0·49) [−9 to 10]	0·0001				
15–18	50	4·2 (0·32) [1–11]	−2·0 (0·55) [−11 to 6]	0·0006	189	103·0 (1·21) [63–168]	8·8 (1·45) [−36 to 80]	<0·0001
18–21	50	4·3 (0·51) [1–19]	−2·1 (0·65) [−11 to 15]	0·0022				
21–24	42	4·6 (0·61) [1–17]	−1·4 (0·64) [−11 to 11]	0·0333	178	104·7 (1·23) [65–169]	10·4 (1·59) [−42 to 81]	<0·0001
24–27	37	4·0 (0·49) [1–13]	−2·9 (0·65) [−11 to 4]	<0·0001				
27–30	30	4·4 (0·95) [1–24]	−1·5 (0·98) [−9 to 18]	0·1298	135	106·7 (1·44) [66–159]	11·1 (1·86) [−30 to 71]	<0·0001
30–33	10	3·1 (0·55) [1–7]	−3·0 (0·65) [−6 to 1]	0·0013				
33–36	7	2·4 (0·30) [2–4]	−3·6 (1·13) [−10 to −1]	0·0196	86	105·6 (1·79) [64–146]	9·5 (2·20) [−31 to 68]	<0·0001

SE, standard error.
*Haemoglobin assessment at the end of corresponding study period.
†Haemoglobin assessment occurred 2 weeks after treatment initiation.

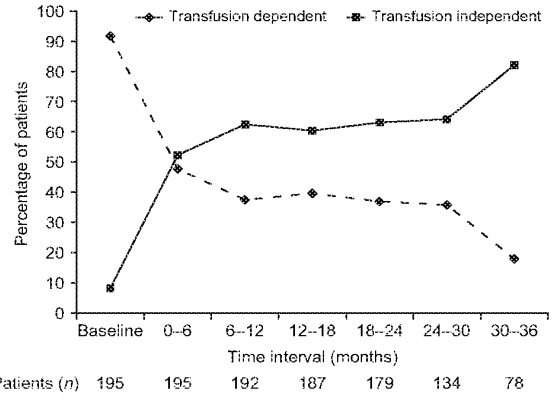


Fig 3. Percentage of transfusion-independent and transfusion-dependent patients over time. Transfusion-independent patients were those who did not require a blood transfusion during the previous 6 months; transfusion-dependent patients had received at least one blood transfusion in the previous 6 months.

median time from the first dose of eculizumab to the first TE was 646 d (approximately 21 months), with a range of 170–876 d (approximately 5·6–29·0 months).

A time-matched analysis showed that in the 467·1 patient-years prior to eculizumab treatment, there were a total of 52 TEs reported in 25 patients. The administration of eculizumab reduced the TE incidence rate from 11·13 events per 100 patient-years to 2·14 events per 100 patient-years, a relative reduction of 81·8% ($P < 0·0005$).

Overall, 98 patients (50·3%) were treated with anticoagulants prior to or coincident with eculizumab: 11 patients before the start of the study, 84 patients both before and during treatment with eculizumab and three patients during the study following a TE. Of the 95 patients who were treated with an anticoagulant prior to the start of the study, 58 (61·1%) experienced at least one TE before receiving eculizumab. Eleven patients, six of whom had a prior history of TEs, discontinued anticoagulant therapy while receiving eculizumab. None of these 11 patients experienced a TE after discontinuation of anticoagulant therapy.

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Chronic kidney disease

Chronic kidney disease stages (National Kidney Foundation, 2002) were assessed following each 6-month eculizumab treatment period. Previous reports on this patient population have demonstrated that prior to eculizumab treatment, 64% of patients had evidence of CKD (Hillmen *et al*, 2010). There was a time-dependent improvement in renal function. The percentage of patients showing improvement, worsening or no change in CKD was 25·4%, 6·1% and 68·5% respectively, at 6 months compared with 44·8%, 6·9% and 48·3% respectively, at 36 months. Overall, following 36 months of treatment with eculizumab, 93·1% of patients showed either an improvement or stabilization in CKD, and patients were significantly more likely to experience an improvement than a worsening in renal function ($P = 0·015$).

Patient survival

Four of the 195 patients died while on eculizumab treatment; the Kaplan–Meier estimate of patient survival at 36 months was 97·6% [95% confidence interval (CI), 93·7–99·1%], which was sustained out to 66 months (Fig 4). Mean duration of therapy with eculizumab was 399 d (range: 60–720 d). Three deaths were caused by concomitant conditions: chronic myelomonocytic leukaemia in a 71-year-old patient who had a history of myelodysplasia and died after 208 d of eculizumab therapy; metastases of pre-existing stomach adenocarcinoma in a 63-year-old patient who died after 609 d of therapy; and cerebral herniation in a 31-year-old patient who died after 60 d of therapy. During in-patient care for a pulmonary embolism, following a series of hospitalizations for a disc prolapse, pneumonia and renal failure, the patient started hyperventilating with his pupils becoming dilated. A computerized tomography scan of the brain revealed a marked left hemorrhagic cerebral arterial media infarct with clear signs of cerebral herniation and the start of cerebrospinal fluid block. The last assessment of LDH levels, almost 4 weeks prior to the event, were below the patient's chronic baseline haemolytic state, leading the investigator to conclude that no serious haemolysis was present at that time.

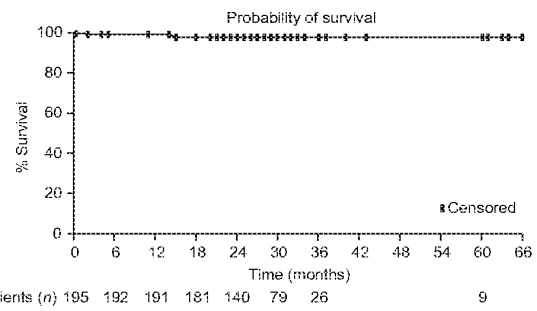


Fig 4. Long-term survival with eculizumab therapy.

The fourth patient, a 35-year-old with unconfirmed aplastic anaemia who had received therapy for 720 d, experienced an arteriovenous thrombosis of the small bowel. The investigator decided to discontinue treatment with eculizumab because of the patient's aplastic anaemia; the patient experienced the TE 19 d after the last eculizumab dose, and died with clinical symptoms of sepsis following resection of the small intestine. Further details relating to this patient have been previously reported (van Bijnen *et al*, 2012). None of the deaths were considered related to treatment with eculizumab.

Safety

Results from this study indicated that eculizumab was well tolerated by patients with PNH. Table III lists AEs that were reported in at least 10% of patients, regardless of relationship to treatment. The most frequently reported AEs were headache, nasopharyngitis and upper respiratory tract infection, each reported in more than 40% of patients. The majority of AEs (91·3%) were mild to moderate in severity.

Table III. Treatment-emergent adverse events reported in ≥ 10% of patients.

Adverse event	Number (%) of patients ($n = 195$)
Patients reporting at least one adverse event	194 (99·5)
Headache	107 (54·9)
Nasopharyngitis	97 (49·7)
Upper respiratory tract infection	80 (41·0)
Diarrhoea	68 (34·9)
Nausea	63 (32·3)
Vomiting	50 (25·6)
Back pain	48 (24·6)
Abdominal pain	43 (22·1)
Arthralgia	43 (22·1)
Oropharyngeal pain	42 (21·5)
Pyrexia	40 (20·5)
Cough	39 (20·0)
Dizziness	39 (20·0)
Pain in extremity	39 (20·0)
Influenza-like illness	34 (17·4)
Urinary tract infection	33 (16·9)
Viral infection	30 (15·4)
Constipation	29 (14·9)
Contusion	29 (14·9)
Myalgia	29 (14·9)
Fatigue	25 (12·8)
Abdominal pain upper	24 (12·3)
Insomnia	23 (11·8)
Sinusitis	23 (11·8)
Epistaxis	21 (10·8)
Oedema peripheral	20 (10·3)
Pruritus	20 (10·3)
Rash	20 (10·3)

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Serious AEs were reported in 75 of the 195 patients (38·5%), with some of the most frequently reported serious AEs, such as haemolysis, abdominal pain and anaemia, typically seen in patients with PNH. These events generally occurred at times of biological stress, most typically in conjunction with an infection. Other serious AEs reported by at least 2% of patients were pyrexia (4·6%) and viral infection (3·1%). Cholecystectomies were reported in six of the 195 patients (3·1%), and one patient developed chronic myelomonocytic leukaemia, resulting in the death of the patient.

Of the 19 patients who discontinued treatment, five (26·3%) discontinued as a result of a nonfatal AE. Two patients became pregnant, one patient developed myelodysplastic syndrome, one patient had meningococcal sepsis (see Table IV) and one patient had, in the opinion of the investigator, a worsening of PNH. This patient suffered from diabetes and had persistent anaemia and impaired renal function, but without reticulocytosis. Of note, three of these 19 patients (15·8%) experienced a TE within 8 weeks of their last dose of eculizumab, with clinical details for one of these patients having been previously described (van Bijnen *et al*, 2012). In the two patients who became pregnant, eculizumab was received for the first 4 and 5 weeks of gestation. Both pregnancies were without complication and resulted in the delivery of healthy babies without any adverse effects from eculizumab treatment (Kelly *et al*, 2010).

Three of the patients included in this study had a history of myelodysplastic syndrome (MDS) prior to treatment with eculizumab. At the time of last follow-up, one patient had spontaneous resolution of the disorder 6 years after onset, one patient was alive and had transitioned to commercially available eculizumab and, as mentioned previously, one patient developed chronic myelomonocytic leukaemia and subsequently died. A fourth patient was diagnosed with MDS during treatment with eculizumab and was withdrawn from the study prior to receiving a bone marrow transplantation; this patient did not show any signs of serious haemolysis in the 12 weeks of follow-up following discontinuation of eculizumab.

Table IV. Serious infection-related treatment-emergent adverse events reported during eculizumab therapy (n = 195).

Adverse event	Number (%) of patients	Mean onset (days)*
Pyrexia	9 (4·6)	446
Viral infection	6 (3·1)	477
Lower respiratory tract infection	3 (1·5)	833
Urinary tract infection	3 (1·5)	878
Cellulitis	2 (1·0)	235
Meningococcal sepsis	2 (1·0)†	385
Pneumonia	2 (1·0)	456
Respiratory tract infection	2 (1·0)	664
Sepsis	2 (1·0)	604
Septic shock	2 (1·0)	312
Viral gastroenteritis	2 (1·0)	419

*Mean interval between date of first eculizumab dose until the adverse event onset date.
†One patient discontinued treatment.

Forty patients (20·5%) reported a total of 67 serious infection-related treatment-emergent AEs, none of which were fatal. Table IV presents all serious infection-related events reported in two or more patients. The most frequently reported events were pyrexia (reported in nine patients) and viral infection (reported in six patients). There was one case of staphylococcal infection, which was successfully treated with cefazolin sodium and two doses of vancomycin, although the patient subsequently withdrew from the study.

Two cases of meningococcal sepsis were reported during treatment, an infection rate of 0·42 per 100 patient-years. One was a serotype B infection that occurred in a 24-year-old male patient 353 d after the initiation of eculizumab. This patient had received a quradivalent vaccine against serotypes A, C, W135 and Y. The infection was successfully treated with several antibiotics, including imipenem, vancomycin, ceftriaxone and penicillin; the range of antibiotics used was because of bacterial sensitivity and the resistance pattern in the local hospital flora. This patient remained in the study and continued to receive eculizumab. The other case occurred in a 54-year-old female patient 416 d after eculizumab treatment was initiated and was due to serotype Y or W135 (further serotyping was not possible in this patient's country). This patient had been vaccinated against serotypes A and C. This infection was successfully treated with meropenem, vancomycin, ceftriaxone and ciprofloxacin, although the patient discontinued treatment on the advice of the investigator. Of note, neither of the patients with meningococcal sepsis had received vaccination against the specific strain of their infection, at the time of the infections serum bactericidal antibody values were within an appropriate range and both infections resolved with treatment and without sequelae. In neither case was the meningococceal infection due to failure of the vaccine.

All AEs coded as pyrexia were considered related to infection even if an identifiable infection was not diagnosed at the time of the fever. Overall, there were 38 events of pyrexia reported in 22 patients, the majority of which (26 of 38; 68·4%) were mild in severity, whereas only three of 38 events (7·9%) were considered severe. As previously mentioned, nine patients (4·6%) had pyrexia events reported as SAEs, which included all the events considered to be severe. All events of pyrexia resolved without sequelae.

Adverse events related to treatment infusion were reported in 71 patients (36·4%). Peripheral oedema, pruritus and rash were the most frequently reported AEs of this type, each occurring in a total of 20 patients (10·3%). None of the AEs associated with treatment infusion resulted in the discontinuation of eculizumab.

In order to investigate if long-term treatment with eculizumab increased the incidence of any individual or category of AE or whether eculizumab was associated with

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Table V. Adverse events reported at a significantly different incidence in the first and last 26 weeks of treatment with eculizumab*.

Adverse event	Number of patients: First 26 weeks	Number of patients: Last 26 weeks	*P* value†
Any event	189	145	<0·001
Headache	87	20	<0·001
Nasopharyngitis	47	32	0·029
Nausea	33	21	0·029
Constipation	14	3	0·006
Back pain	20	9	0·031
Arthralgia	17	8	0·032
Muscle spasms	9	0	0·002
Dizziness	17	8	0·039
Pyrexia	18	9	0·032
Epistaxis	12	4	0·011
Fatigue	12	4	0·029
Insomnia	10	2	0·019
Respiratory tract infection	7	0	0·008
Oral herpes	8	1	0·020
Chest pain	6	1	0·031
Haematoma	6	1	0·031
Lethargy	5	0	0·031
Toothache	5	0	0·031

*For patients who received eculizumab for a total of <52 weeks, the incidence of adverse events in the first 26 weeks was compared with the period from the start of the 27th week until the last dose of treatment. Only events for which there was a statistically significant difference between the treatment periods are reported.

†*P* values determined from one-tailed exact McNemar test for matched-pairs data.

cumulative toxicity, the incidence of AEs (irrespective of relation to treatment) reported during the first 26 weeks of eculizumab treatment was compared with the last 26 weeks of treatment (Table V). Overall, significantly fewer patients reported at least one AE in the last 26 weeks of treatment ($n = 145$) than in the first 26 weeks of treatment ($n = 189$; $P < 0·001$), and no individual AE was reported by a statistically significantly greater number of patients during the last 26 weeks than during the first 26 weeks of treatment with eculizumab. Although this analysis included patients who had a total treatment duration of <52 weeks, the mean (median) observation times of 26·00 (26·0) and 25·86 (26·0) weeks for the first and last observation periods respectively, indicated that the reduction in number of AEs seen in the last 26 weeks of therapy was not due to the reporting of AEs over a shorter observation time.

Assessments of clinical laboratory tests and vital signs did not reveal any consistent or clinically significant mean changes or any obvious treatment effect on these parameters. There were small increases in direct bilirubin concentrations, although median levels of total bilirubin remained essentially unchanged.

Discussion

The results from this study demonstrate the long-term safety and efficacy of chronic eculizumab treatment in a large cohort of patients with PNH ($n = 195$). The median eculizumab treatment duration was 30·3 months, with a maximum duration of 66 months. Inhibition of terminal complement activity with eculizumab has previously been reported to rapidly and significantly reduce chronic haemolysis, leading to reductions in transfusion requirements (Hillmen *et al*, 2006) and TEs (Hillmen *et al*, 2007), as well as improvements in renal function (Hillmen *et al*, 2010). The current study shows that these results are sustained with long-term treatment.

Thrombotic events are a severe and life-threatening complication of PNH and are reportedly the most frequent cause of death in patients with PNH (Hillmen *et al*, 2007). TE has been shown to be a significant risk factor for early mortality (relative risk: 15·4; 95% CI, 9·3–25·4; $P < 0·001$) in untreated patients (de Latour *et al*, 2008). Furthermore, following the occurrence of a first TE, there is a greater risk of subsequent TEs, which places patients with a history of TE at a heightened risk of premature mortality (Nishimura *et al*, 2004; de Latour *et al*, 2008). Given that TE in PNH patients appears to be related to severe and ongoing uncontrolled terminal complement activation, management of TE is difficult in PNH patients, as many continue to report TE despite prophylactic use of anticoagulation therapies (Moyo *et al*, 2004; de Latour *et al*, 2008; Kelly *et al*, 2011). We observed an 81·8% reduction in the incidence of TEs with long-term eculizumab treatment. Eighty-four patients in this study received concomitant anticoagulant therapy, which was discontinued in 11 patients. Despite six of these 11 patients having a history of TE, none of them experienced a TE following withdrawal of anticoagulation therapy while receiving eculizumab alone. This experience is consistent with other reports (Emadi & Brodsky, 2009; Kelly *et al*, 2011).

Thrombotic events were observed in 16% (3 of 19) of patients who discontinued treatment with eculizumab, all within 8 weeks of taking their last dose. This finding highlights the importance of adherence to eculizumab treatment at the recommended dose and schedule over the long term, as continuous therapy is paramount in maintaining sustained inhibition of the underlying uncontrolled terminal complement activation. If discontinuation of eculizumab therapy becomes necessary, the potential risk of thrombosis as well as possible preventive measures should be considered.

Chronic kidney disease in PNH patients is caused by several factors, including chronic exposure to free haemoglobin, which increases renal accumulation of haemosiderin, tubulointerstitial inflammation and kidney damage (Nath *et al*, 2001). Renal impairment is known to progress over time (Hillmen *et al*, 2010) and is associated with an increase in mortality (Kim *et al*, 2011). Continued improvement in CKD was observed with sustained eculizumab treatment,

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with 44·8% of patients showing improvement after 36 months of treatment, compared with 24·4% showing improvement in CKD after only 6 months of treatment. The improvement in renal function in all nine patients with early-stage CKD (stage 1–2) over 36 months suggests that patients treated with eculizumab at earlier stages of kidney damage have a greater likelihood of improvement in renal function, and there is evidence that sustained long-term therapy with eculizumab can reverse early stage renal damage sustained prior to treatment initiation. These are unique findings in a patient population known to be at risk for progressive decline in renal function (Hillmen *et al*, 2010), and they suggest that eculizumab may have a protective role that can lead to improvement in, or less progression of, renal disease.

Reduction in the incidence of TE and CKD, the two major causes of death in PNH, by administration of eculizumab would be expected to lead to improved patient survival. In the current prospective, multinational study in a large cohort of 195 patients, we demonstrated a patient survival of 97·6% (95% CI, 93·7%–99·1%) at 3 years, which was maintained over 66 months of eculizumab treatment. This result is similar to the 5-year survival rate of 95·5% observed in a single-centre, retrospective analysis of 79 patients (Kelly *et al*, 2011). These survival rates seen with eculizumab therapy are a substantial improvement over historical 5-year survival rates of approximately 65% in PNH patients with evidence of haemolysis (Hillmen *et al*, 1995; Kelly *et al*, 2011) and are similar to survival rates of age- and sex-matched healthy subjects (Kelly *et al*, 2011). These results further emphasize the role of uncontrolled complement activation in the pathophysiology of PNH-related mortality.

Long-term treatment with eculizumab also resulted in sustained haematological improvement in patients. Transfusion independence was achieved by 52% (102 of 195) of patients within the first 6 months of treatment, which increased to 82% by the last 6 months of treatment. The number of units of PRBCs transfused was reduced by 55% over the 36-month period. In addition, there was a continuous improvement in haemoglobin levels, which was achieved despite the reduction in transfusions.

The standard eculizumab maintenance dose of 900 mg every 14 ± 2 d was sufficient to maintain inhibition of haemolysis in 89% of patients. In the 21 patients who did report breakthrough haemolysis, shortening the dosing interval to <14 d was sufficient to maintain inhibition of haemolysis, and none of these patients discontinued treatment because of a lack of therapeutic effect. It is important that patients be monitored for any signs or symptoms of breakthrough haemolysis so that the dosing can be adjusted accordingly or, in cases of persistent breakthroughs, to ensure the patient has not developed human anti-human antibodies (HAHA) or neutralizing antibodies to eculizumab. Subsequent to the end of the study, a more sensitive analysis became available for the identification of HAHA in patients receiving eculizumab. With this new analysis, two of the patients in the long-term extension study were found to have low positive values for neutralizing antibodies, which exceeded the prespecified threshold for the *in vitro* assay (data not shown). This finding had no impact on the clinical response to eculizumab therapy or on the pharmacokinetic and pharmacodynamic effects of eculizumab, which continued to block complement activity.

Eculizumab was well tolerated over the long term and demonstrated a positive safety profile. Although nearly all patients reported at least one AE, this is to be expected considering the duration of the study; importantly, discontinuation from treatment due to a nonfatal AE was seen in only five patients (2·6%) over the entire period of study. With no comparator group in this open-label extension, we compared the AE rate of the early treatment group with that of the later treatment group to understand the risk of long-term eculizumab treatment. The probability of a patient experiencing one or more AEs was significantly lower in the last 6 months of the eculizumab treatment period than in the first 6 months of therapy. This suggests that there is no cumulative toxicity with long-term administration of eculizumab and that the risk–benefit profile of eculizumab improves with extended use. Serious infection-related AEs were observed throughout the study, although there was no evidence that their occurrence was related to time on eculizumab treatment.

There is an increased incidence of infections with *N. meningitidis* in individuals with genetic deficiencies of C5–C9 (Figueroa & Densen, 1991). Thus, blocking terminal complement activity with eculizumab increases a patient's susceptibility to meningococcal infections. Two cases of meningococcal sepsis were reported during the approximately 467 patient-years of exposure to eculizumab. Both patients were successfully treated without sequelae. Patient education on the signs and symptoms of potential meningococcal infections remains important to ensure that any infections are identified as quickly as possible. This is particularly crucial outside of a closely monitored clinical study, where continuous vigilance and proactive antibiotic treatment are essential to avoid a potentially fatal infection (http://soliris.net/sites/default/files/assets/soliris_pi.pdf).

In conclusion, long-term treatment with eculizumab resulted in sustained improvement in patient outcomes by rapidly reducing haemolysis and significantly reducing the frequency of severe and life-threatening morbidities, such as TEs and CKD, and thus, improving patient survival. There was evidence that eculizumab improved kidney function over time and also provided sustained protection against further renal damage, particularly when administered early and prior to advanced renal impairment. Eculizumab therapy was well tolerated with a favourable safety profile; very few patients discontinued treatment during long-term therapy. Over the course of this study, survival

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rates were high and consistent with the improvements observed in another overlapping cohort of patients in whom survival was seen to be similar to age- and sex-matched normal control subjects (Kelly *et al*, 2011). The International PNH Registry (www.pnhregistry.com) is providing further opportunities to continue studying and understanding the long-term benefits of eculizumab in patients with PNH outside the clinical trial setting.

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Authorship contributions

All authors participated in study design, collection, analysis and interpretation of the data presented in this study, review and critical appraisal of the contents of manuscript at each stage of its preparation and provided final approval of the completed manuscript.

Conflicts of interest

P.H. served on an advisory committee for and received honoraria from Alexion Pharmaceuticals. P.M. served on an advisory committee for Alexion Pharmaceuticals. A.R. served on an advisory committee for and received honoraria and research funding from Alexion Pharmaceuticals. M.O.E. served on an advisory committee for and received honoraria from Alexion Pharmaceuticals. A.M.R. served on an advisory committee for and received honoraria from Alexion Pharmaceuticals. H.S. served on an advisory committee for and received honoraria and research funding from Alexion Pharmaceuticals and received honoraria from Genzyme. J.S. served as a consultant for and received honoraria from Alexion Pharmaceuticals, Ortho Biotech and Roche. P.B. has nothing to disclose. J.P.M. served as a consultant for Alexion Pharmaceuticals and received research funding from Celgene and Eisai. J.S. served on an advisory committee for and received honoraria from Alexion Pharmaceuticals and served on advisory committees for Celgene, Novartis and Pfizer. A.U.-I. served on an advisory committee for Alexion Pharmaceuticals. C.D. served on a speakers bureau for Alexion Pharmaceuticals. G.S. served as a consultant for Alexion Pharmaceuticals and served on advisory committees for and received honoraria from Therakos and Fresenius. R.A.B. served on an advisory committee for Alexion Pharmaceuticals.

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NATURAL HISTORY OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

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Abstract *Background.* Paroxysmal nocturnal hemoglobinuria (PNH), which is characterized by intravascular hemolysis and venous thrombosis, is an acquired clonal disorder associated with a somatic mutation in a totipotent hematopoietic stem cell. An understanding of the natural history of PNH is essential to improve therapy.

Methods. We have followed a group of 80 consecutive patients with PNH who were referred to Hammersmith Hospital, London, between 1940 and 1970. They were treated with supportive measures, such as oral anticoagulant therapy after established thromboses, and transfusions.

Results. The median age of the patients at the time of diagnosis was 42 years (range, 16 to 75), and the median survival after diagnosis was 10 years, with 22 patients (28 percent) surviving for 25 years. Sixty patients have died; 28 of the 48 patients for whom the cause of death is known died from either venous thrombosis or hemorrhage. Thirty-one patients (39 percent) had one or more episodes of venous thrombosis during their illness. Of the 35 patients who survived for 10 years or more, 12 had a spontaneous clinical recovery. No PNH-affected cells were found among the erythrocytes or neutrophils of the patients in prolonged remission, but a few PNH-affected lymphocytes were detectable in three of the four patients tested. Leukemia did not develop in any of the patients.

Conclusions. PNH is a chronic disorder that curtails life. A spontaneous long-term remission can occur, which must be taken into account when considering potentially dangerous treatments, such as bone marrow transplantation. Platelet transfusions should be given, as appropriate, and long-term anticoagulation therapy should be considered for all patients. (N Engl J Med 1995;333:1253-8.)

PAROXYSMAL nocturnal hemoglobinuria (PNH) is an acquired disorder of hematopoiesis characterized by intravascular hemolysis and manifested by episodes of hemoglobinuria and life-threatening venous thromboses.[1] The cellular abnormality in this disorder is caused by a somatic mutation in a totipotent hematopoietic stem cell.[2,3] The cells derived from the abnormal clone are deficient in all surface proteins normally attached to the cell membrane by a glycosylphosphatidylinositol anchor.[4,5]

PNH was first described as a distinct clinical entity in 1882.[6] The cardinal diagnostic test, introduced in the late 1930s, is Ham's test,[7] which is based on the increased sensitivity of PNH-affected erythrocytes to lysis by complement. Deficiency of an antigen known as the membrane inhibitor of reactive lysis (CD59) is largely responsible for the hemolysis[8,9] and is implicated in the tendency for patients to have thromboses.[9] In the past two years the biochemical defect underlying PNH has been pinpointed at an early step in the biosynthesis of glycosylphosphatidylinositol molecules — namely, the transfer of *N*-acetylglucosamine to phosphatidylinositol.[10-12] The protein required for this step is encoded by a gene, *PIG-A*, that is somatically mutated in patients with PNH.[13-17]

Despite the remarkable progress in our understanding of this disorder, treatment has remained largely supportive. The only potentially curative therapy currently available is bone marrow transplantation,[18,19] which is associated with substantial morbidity and mortality.[20] To determine whether the management of PNH can be improved, it is important to know its natural history. We report the results of a long-term study (up to 48 years after the diagnosis) of a group of 80 patients with PNH seen at one institution between 1940 and 1970. This study has uncovered some surprising information on the natural history of the disorder and has identified relatively simple therapies that may reduce the associated morbidity and mortality.

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METHODS

Patients

Eighty patients with PNH were referred to Hammersmith Hospital in London between 1940 and 1970. In all patients the diagnosis was established or confirmed by a positive Ham's test.[21] This group of patients was last described in 1972.[22] Follow-up data have been obtained by contacting the patients' primary physicians and reviewing death certificates.

Complications and Causes of Death

The complications and causes of death reported were either unequivocally diagnosable on clinical grounds or were diagnosed after death. If a complication was not confirmed, it is not reported here. Thus, the reported incidence of complications is likely to be an underestimate, because many of the patients were seen at Hammersmith Hospital only infrequently. We compared the survival of the patients with the survival of a control group matched for sex and age.

Analysis of Blood Samples

In 1993 venous blood samples were obtained from five patients in either acid–citrate–dextrose or EDTA for the performance of Ham's tests and flow cytometric studies of the expression of glycosylphosphatidylinositol-linked proteins on erythrocytes and white cells, as previously described.[23,24] By definition, normal cells express normal levels of glycosylphosphatidylinositol-linked proteins, whereas PNH-affected cells are deficient in all these proteins.

RESULTS

Follow-up Studies

Sixty of the 80 patients were known to have died, and 6 patients were lost to follow-up before 1972. The remaining 14 patients were alive when last seen between

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1975 and 1994, and 9 of the 14 were known to be alive in 1994.

Clinical Presentation

The presenting features or initial diagnoses were reported previously.[22] The median age at the time of diagnosis was 42 years (range, 16 to 75). Forty-three (84 percent) of the 51 patients for whom reliable information was available had episodes of hemoglobinuria as a chief symptom at some time during their illness.

Course of Illness and Survival

The course of the disease in each of the 80 patients is shown in Figure 1. From the actuarial survival curve for the group, we calculated a median survival of approximately 10 years (Fig. 2). Twenty-five years after the diagnosis, 58 patients (72 percent) had died, and 22 (28 percent) were alive. The median age at the time of death was 56 years (range, 20 to 84), with a median interval of 10 years (range, 0 to 48) between diagnosis and death. Patient 10 received the diagnosis of PNH in 1939, had a spontaneous remission in 1949, and died of bronchial carcinoma in 1987, 48 years after the diagnosis.

Cause of Death

For 12 of the 60 patients who died (Table 1), the cause of death was unknown. In 28 of the other 48 patients (58 percent), the cause of death (either thrombosis or hemorrhage attributable to thrombocytopenia) was directly related to PNH. In the other 20 patients (42 percent), the cause of death was unrelated to PNH.

Venous Thrombosis

Thirty-one patients (39 percent) were known to have had one or more episodes of venous thrombosis during the course of their illness (Table 2), and several patients had repeated thromboses. In many patients the thrombosis was either fatal or life-threatening, and it was often unheralded.

Spontaneous Remission

Twelve patients (15 percent) had spontaneous clinical remissions (Fig. 1), with negative Ham's tests in all nine who were tested. We later obtained blood samples from five of these nine patients; in all five the erythrocytes expressed normal levels of glycosylphosphatidylinositol-linked proteins, with no demonstrable PNH-affected erythrocytes (Fig. 3A). In addition, of the four patients tested, none had PNH-affected neutrophils (Fig. 3A), but three of the four had small numbers of PNH-affected lymphocytes (Fig. 3B). Further analysis by double fluorescent staining demonstrated a subpopulation of PNH-affected lymphocytes among CD4+

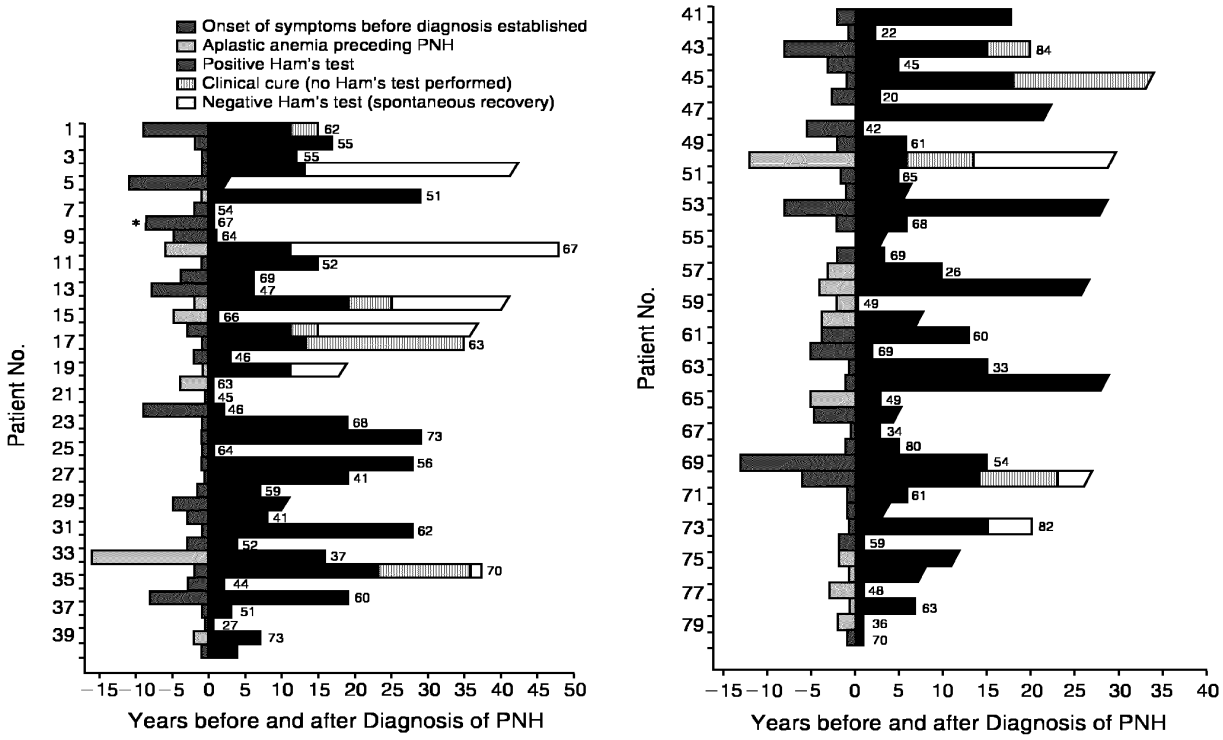


Figure 1. Course of Illness in 80 Consecutive Patients with PNH.

The x axis indicates the years before and after the diagnosis of PNH, which is denoted by the zero on the axis. Bars ending in a straight line indicate patients who died, and their ages at death are shown; bars ending in a diagonal line indicate surviving patients. Nine patients had complete clinical recovery, with negative Ham's tests. Three additional patients had protracted clinical remissions, but Ham's test was not repeated during remission. The asterisk indicates a prolonged but unspecified period of symptoms before the diagnosis was established.

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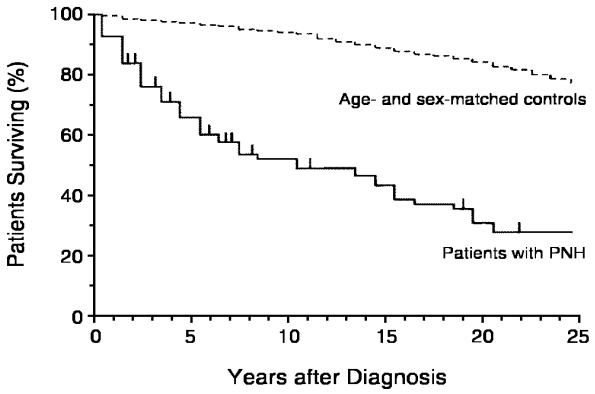


Figure 2. Actuarial Survival from the Time of Diagnosis in 80 Patients with PNH.

The median survival was 10 years. The expected survival of an age- and sex-matched control group is shown for comparison.

T cells, CD8+ T cells, and CD19+ B cells in all three patients.

Associated Disorders

Twenty-three patients (29 percent) received a diagnosis of aplastic anemia before the diagnosis of PNH. Nine of these patients subsequently had hemolytic PNH, with episodes of overt hemoglobinuria, and six did not; whether the remaining eight patients ever had hemoglobinuria could not be reliably determined.

At the time of the diagnosis, 64 of the 80 patients (80 percent) had thrombocytopenia (platelet count, <150,000 per cubic millimeter), and 38 patients (48 percent) had severe thrombocytopenia (platelet count, <50,000 per cubic millimeter). In addition, 44 patients (55 percent) had neutropenia (neutrophil count, <1500 per cubic millimeter).

In five patients the disorder progressed from hemolytic PNH to aplasia. Ham's test remained positive in four of these patients, but the fifth (Patient 34) subsequently had a negative Ham's test, with no PNH-affected erythrocytes or neutrophils. The patient died from bleeding due to severe thrombocytopenia 37 years after the diagnosis of PNH and at least 8 years after Ham's test had become negative.

Leukemia was not known to have developed in any of the patients.

Discussion

Clinical Features

In this group of 80 patients who were followed for up to 48 years, the median actuarial survival was 10 years, with 28 percent of the patients surviving for 25 years. Death was directly attributable to PNH or to bone marrow hypoplasia in 58 percent of the patients who died. Therapy with platelet concentrates should reduce mortality from hemorrhage due to thrombocytopenia.

At least 39 percent of the patients had venous thrombosis at some time during their illness. Many patients had initial thromboses that were life-threatening, but two (Patients 17 and 70), who probably had postpartum hepatic-vein thrombosis, recovered and survived for 34 and 27 years, respectively, after the thrombosis. One other patient (Patient 34) survived for 24 years after a thrombosis of the inferior mesenteric vein.

Spontaneous Remission

In some patients with PNH, the severity of the illness diminishes with time,[25] and some patients have a complete clinical remission,[22,26] although laboratory abnormalities may persist for years.[27] In this series 15 percent of the patients had spontaneous clinical remissions, and Ham's test became negative in the patients who were tested. Neither the severity of symptoms or complications nor the proportion of red cells lysed in Ham's test was correlated with an eventual remission. Among the patients with remissions, one (Patient 14) had severe, transfusion-dependent PNH and thromboses, and at

Table 1. Causes of Death in 60 Patients with PNH.

Cause of Death	No. of Patients
Probably related to PNH	
Venous thrombosis	
Hepatic vein	7
Inferior vena cava	1
Cerebral vein	1
Mesenteric vein	2
Pulmonary embolism	3
Hemorrhage	
Gastrointestinal	6
Subarachnoid	3
Intracerebral	2
Miscellaneous	
Liver failure	2
Intraabdominal event	1
Probably unrelated to PNH	
Arterial thrombosis	
Myocardial infarction	6
Cerebrovascular accident	2
Bronchopneumonia	3
Chronic obstructive airways disease plus cor pulmonale	2
Cardiac tamponade	1
Constrictive pericarditis	1
Renal failure	1
Amyloidosis	1
Lymphoma	1
Bronchial carcinoma	1
Epilepsy	1
Unknown	12

Table 2. Sites and Types of Thrombosis.

Site and Type of Thrombosis	No. of Patients
Intraabdominal	
Hepatic vein	8
Inferior vena cava	3
Mesenteric vein	4
Splenic vein	1
Renal vein	1
Unspecified	1
Other venous sites	
Cerebral vein	4
Pulmonary embolism	9
Deep vein	7
Superficial	3
Arterial	
Myocardial infarction	6
Cerebrovascular accident	2

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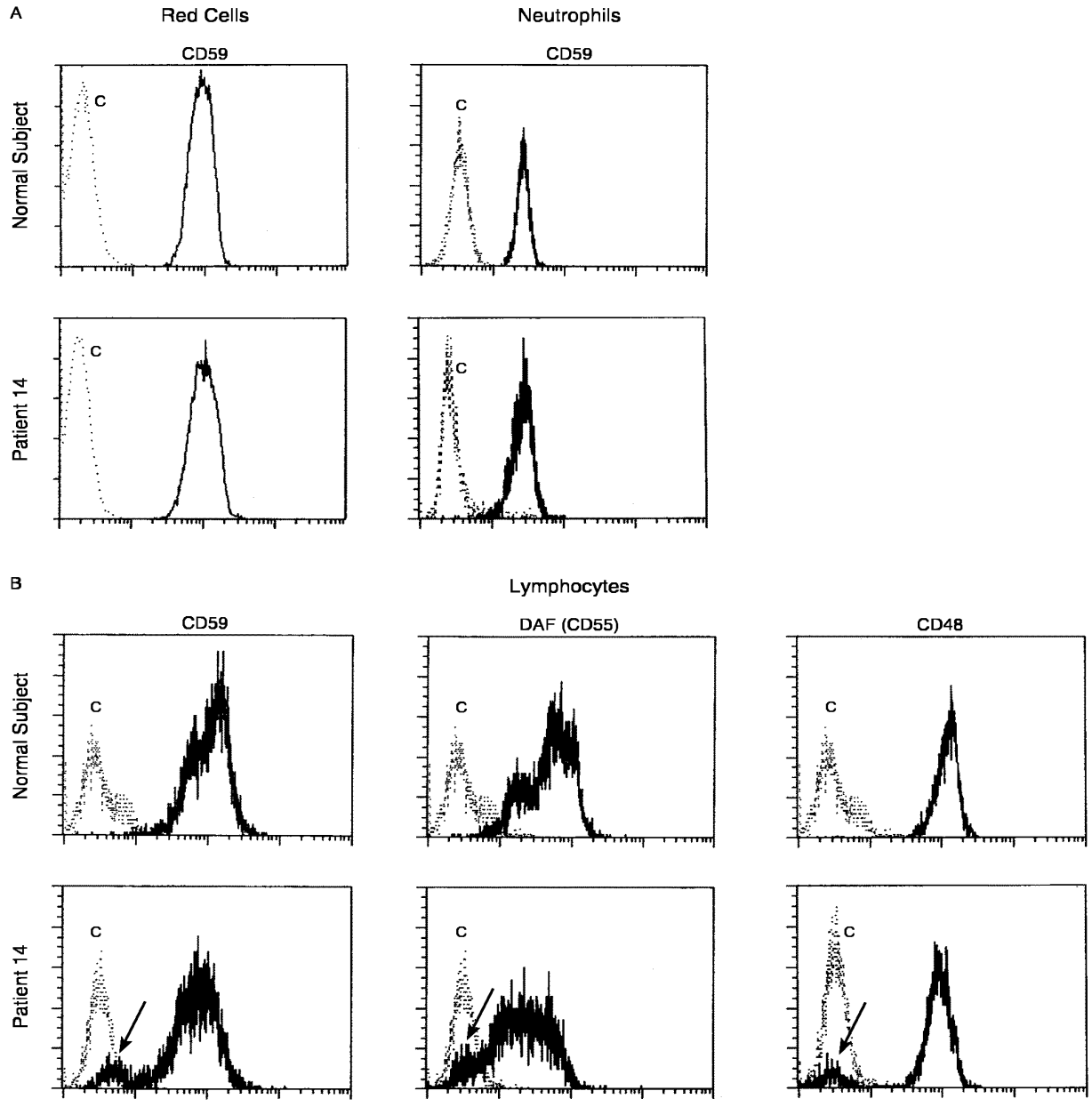


Figure 3. Flow-Cytometric Analysis of Blood Cells from a Normal Subject and Patient 14 after Spontaneous Recovery from PNH.

The analysis was carried out 20 years after the patient's clinical remission began and 17 years after her Ham's test had become negative. Panel A shows a unimodal distribution of cells with normal reactivity after reaction with anti-CD59 antibody (a molecule absent from the surface of PNH-affected cells). The distributions obtained with the patient's red cells and neutrophils are indistinguishable from those of the cells from a normal subject, demonstrating that no PNH-affected cells remain. C indicates distributions obtained in control experiments with an antibody directed against a molecule absent from normal blood cells.

Panel B shows the results of an analysis, similar to that in Panel A, carried out on peripheral-blood lymphocytes. The distributions of cells from a normal subject show that, as compared with the results of analyses with negative control antibody (C), all the lymphocytes reacted with all three antibodies used. The distribution is unimodal for CD48 and bimodal for CD59 and CD55. In contrast, the cells from Patient 14 have a small population of lymphocytes that are unreactive with all three antibodies (arrows), which indicates that these cells lack the corresponding antigen protein molecules on their surfaces. The fact that there is a peak of antigen-negative cells for three different proteins that are normally attached to the cell membrane by a glycosylphosphatidylinositol anchor means that the anchor is absent, which is characteristic of the PNH phenotype. The finding that the PNH clone was still present when there were no PNH-affected erythroid or myeloid cells in Patient 14 during a clinical and hematologic remission is consistent with the notion that this clone does not have a selective advantage once normal hematopoiesis has resumed. DAF denotes decay-accelerating factor.

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least two had over 50 percent lysis in earlier Ham's tests, suggesting that they had few residual normal stem cells at the height of their illness. The spontaneous remissions occurred between 10 and 20 years after the diagnosis of PNH. Of the 35 patients who survived 10 years or more, 12 (34 percent) eventually had remissions. A possible explanation for spontaneous remission is that the clones of PNH-affected cells have a finite life span, like normal somatic cells. Recovery may thus depend on the presence of normal stem cells capable of repopulating the bone marrow.[28]

There were no erythrocytes or neutrophils affected by PNH after recovery, but three of four patients tested had some residual abnormal lymphocytes. These results are consistent with the fact that lymphocytes have a much longer life span than myeloid and erythroid cells and that committed lymphocyte progenitor cells undergo division less frequently than the other cells. If the aging process postulated as being responsible for the gradual disappearance of clones of PNH-affected cells is related to the number of cell divisions, this may explain why abnormal lymphocytes persist for many years after the disappearance of abnormal neutrophils and erythrocytes.

PNH and Bone Marrow Failure

The data on this series of patients provide further evidence of the close relation between PNH and aplastic anemia. The coexistence of the two conditions in one patient was first reported in 1944,[29] with subsequent reports in 1952[30] and 1961.[31] Cultures of peripheral blood and bone marrow from patients with PNH consistently show reduced numbers of erythroid progenitor cells (erythroid burst-forming units) and myeloid progenitor cells (granulocyte–macrophage colony-forming units), even in the absence of pancytopenia,[32,33] as in patients with aplastic anemia.[34] In addition, PNH eventually develops in 10 to 31 percent of patients with aplastic anemia treated with immunosuppressive agents.[35] It is important to emphasize that PNH is not a complication of immunosuppressive treatment. Instead, the majority of patients with this disorder probably have an underlying aplastic process. Clones of PNH-affected cells may thus have a relative growth or survival advantage over the residual normal bone marrow in patients who have aplastic anemia.[1,28,36] It is conceivable that the mutation leading to the development of PNH occurs quite frequently but that, in the absence of marrow hypoplasia, the clone has difficulty establishing itself.[28]

PNH and Leukemia

Because of the reports of myelodysplasia and leukemia[1] — invariably, acute myeloid leukemia — in patients with PNH, it has been regarded as a preleukemic condition. Leukemia did not develop in any of our 80 patients, however, indicating that it is relatively rare in unselected patients with PNH. Acute myeloid leukemia eventually develops in approximately 5 percent of patients with aplastic anemia who survive the marrow aplasia.[35] Thus, aplastic anemia may predispose patients to both PNH and acute myeloid leukemia, but the development of PNH may not add to the risk of leukemia associated with uncomplicated aplastic anemia.

In most reported cases of leukemia in patients with PNH in whom the leukemic cells were studied,[37,38] the leukemic clone was derived from the PNH clone. According to a recent report of a patient with PNH who subsequently had a myelodysplastic syndrome, the blasts expressed normal levels of glycosylphosphatidylinositol-linked proteins,[39] indicating that the leukemic event may occur in the residual normal stem cells.

Management

The natural history of PNH is an important factor in decisions about therapy for individual patients. Thrombolytic treatment of hepatic-vein thrombosis with tissue plasminogen activator has been reported to be effective.[40] Oral anticoagulation therapy after venous thrombosis has been used since the 1950s.[25] In view of the high incidence of potentially fatal thrombosis, a strong case can be made for prophylactic anticoagulation treatment in all patients with PNH in whom there is no contraindication (such as severe thrombocytopenia). This approach may improve survival and reduce morbidity.

The only curative therapy at present is bone marrow transplantation, but it is available to only a small proportion of patients and is associated with substantial morbidity and mortality. The recent advances in the understanding of the pathogenesis of PNH and, in particular, of the molecular lesion open opportunities to explore new treatments, such as gene therapy. The possibility of spontaneous remission and of long-term survival must be taken into account when considering either bone marrow transplantation or other new treatments for this disorder.

We are indebted to the late Miss Eleanor Lloyd for her assistance in this project, to the patients, and to the physicians who originally referred the patients and provided follow-up data.

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Immunogenicity of engineered antibodies

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Abstract

Administration of a therapeutic antibody can lead to an anti-antibody response (AAR). Much effort has been applied to engineering antibodies with as little as possible non-human structure to minimize such responses. Here, we review reported AAR to murine, mouse–human chimeric, and humanized antibodies. Replacement of mouse immunoglobulin constant regions with human ones effects the largest immunogenicity reduction. Humanization of variable domains effects a further decrease.
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Keywords: Chimeric antibody; Humanized antibody; Immunogenicity; Human anti-mouse antibody

1. Introduction

Murine monoclonal antibodies (mAbs) have proved tremendously useful in diagnostics. However, when used in the treatment of patients with various ailments, their effect is not always sustained. This is often due to the development of human anti-mouse antibodies (HAMA), leading to clearance of the murine mAb and adverse events that are sometimes fatal.

HAMA have been the impetus for efforts over the last 20 years to reduce the murine content of therapeutic mAb. Chimeric mAb, with human constant regions, humanized mAb, retaining only murine CDRs, and "fully human" mAb made from phage libraries or transgenic mice have progressively reduced the murine content of therapeutic mAb to nil. Publication of this volume on engineered mAb offers an opportunity to review the degree to which the methods for replacement of murine content with human has translated to a corresponding reduction in incidence of anti-antibody response (AAR).

Comparison of the relative immunogenicity of any two antibodies is rife with logical flaws. In general, one would be comparing two structurally different mAb, recognizing different Ag, used for different indications, among different patient groups, on different dosing regimens, with immunogenicity scored by different procedures. Indeed, a landmark study of a humanized anti-tumor necrosis factor α mAb in healthy volunteers [1] demonstrated human anti-humanized antibody (HAHA) activity in *all* subjects in a low dose cohort and *no* subjects in a high dose cohort. While acknowledging the intellectual hazard of AAR's profound dependency on treatment context, we embarked on a review of the literature on AAR to attempt to discern any broad trends in immunogenicity that may distinguish therapeutic antibodies' molecular format.

2. Methods

To review HAMA responses, a Medline search for the period January 1984 to December 2003 using the unrestricted keywords "human AND anti-mouse AND antibodies" or "HAMA" was done. Similarly, review of HACA responses utilized the keywords "human AND

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anti-chimeric AND antibodies" or "HACA" while review of HAHA responses utilized the keywords "human AND anti-human AND antibodies" or "HAHA". Full-length articles were selected for review based on the contents of their published abstracts. Criteria used to select reports for our database analysis were a clear description of AAR, sufficient numbers of patients for analysis and consistency of data between reports. Where there was more than one paper reporting AAR, the paper with either more patients or most representative data were chosen for inclusion in our database for analysis. We also examined prescribing information posted on-line by mAb manufacturers. Additional Medline searches were focused on the keywords "immunogenicity AND antibodies" and "humanization AND immunogenicity." Relevant publications referenced in the reviewed literature were further included for review, if not present in the original Medline search. For antibodies that had obtained approval by the United States Food and Drug Administration (FDA), the relevant company product information was preferentially used.

3. Findings

The results of this study were limited by varied sensitivity and specificity of assays used for the assessment of AAR and varied methods of sample handling and timing of sample collection. We chose to use AAR incidence figures quoted in the literature at face value, without trying to parse patient subsets or intensity of AAR in individual patients. We grouped incidence into three operational categories: *negligible*, *tolerable*, and *marked*. Abs were classified as having *negligible* immunogenicity when AAR was reported in less than 2% of patients. We adopted this classification because immunogenicity this low represents an ideal, with hardly any need for concern about safety. We classified immunogenicity as *tolerable* if detectable in 2–15% of patients. When classified as tolerable, the mAbs were essentially flawed, though use was arguably warranted for catastrophic or life-limiting disease. We classified immunogenicity as *marked* if present in more than 15% of patients. Products with *marked* AAR were usually clinical failures and regulatory concerns were likely to preclude clinical use except for one time use as radioimmunoconjugates. We used these operational classifications to bin the immunogenicity of mAb in the clinic or in clinical development.

3.1. Are mouse mAb immunogenic?

The immunogenicity of mouse antibodies is well known. The compilation of results in Table 1 represents our attempt to define a baseline from which the effect of chimerization or humanization can be compared. Forty-four murine mAb were included in our analysis (Table 1). As seen in Fig. 1, 84% of mAbs had *marked* HAMA reactions, and 7% had *tolerable* HAMA, while 9% had *negligible* HAMA. Of those with negligible HAMA, two of four were used for imaging, not therapy. Of these 44 murine mAb, three (Muromomab-CD3, Ibritumomab, and Tositumomab) were approved by the FDA for marketing. Ibritumomab (Zevalin) and Tositumomab (Bexxar) are both labeled with radioactive isotopes. One other murine antibody (Edrecolomab) was approved for use in Germany and four others are in late stage development/phase III clinical trials. Of the four antibodies approved for marketing, AAR was on the high end for those approved earlier (88% for muromonab-CD3 and 100% for edrecolomab, approved in 1986 and 1995, respectively). For the ones approved in more recent years, AAR was in the lower range for those that obtained approval (<2% for Y90 ibritumomab and 8% for I131 tositumomab, approved in 2002 and 2003, respectively). In view of the high incidence of AAR with murine mAb, the substantial degree of immunogenicity of mouse mAbs is unequivocal. It is very rare for a murine mAb to show negligible immunogenicity, and very common for 100% of patients to show HAMA.

3.2. Are chimeric mAb less immunogenic than mouse mAb?

Fifteen chimeric Abs with clearly reported AAR were included in our analysis (Table 2). We found that 40% had HACA defined as *marked*, 27% *tolerable*, and 33% *negligible* (Fig. 1). Clearly, when the Fc regions of murine mAbs are substituted with human sequences, the incidence of human AAR is much reduced. Of these 15 chimeric mAbs, four have been approved by the FDA for marketing (rituximab, infliximab, cetuximab, and basiliximab) and two more are in phase II clinical trials (SGN30 (no AAR data found) and G250).

3.3. Are humanized mAb less immunogenic than chimeric mAb?

Twenty-two humanized mAb had clearly reported AAR and were included in our analysis (Table 3). Nine percentage had HAHA defined as *marked*, 36% had *tolerable* AAR, and 55% had *negligible* AAR (Fig. 1). Where two versions of the mAb were used the one reporting the higher AAR was used in our analysis. Six of these mAbs (30%) were approved by the FDA for marketing and four more are in late stage development. Two findings emerge from these data. First, the low immunogenicity of humanized antibodies cannot be assumed. For example, humanized mAb A33, used in colon cancer patients [2], had a HAHA incidence of 66%, a value that would be typical for a fully murine mAb. Second, the main impact of humanizing on immunogenicity is reduction of the *marked* category. Humanized mAb with *marked* immunogenicity are notably less

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Table 1
Reported human anti-mouse antibody (HAMA) responses

Antibody (synonyms)	Indication	% of patients with AAR	References
2H4 and 5D3	Nasopharyngeal carcinoma	100	[8]
Anti-Lym1 mAb	B-cell malignancies	38	[9]
10-3D2	Breast tumor	14/indium-111-labeled	[10]
131I-labeled anti-HCC mAb/Hepama-1	Hepatocellular carcinoma	34	[11]
131I-T101	Cutaneous T-cell lymphoma	100/I131-labeled	[12]
14G2a	Refractory melanoma, neuroblastoma, or osteosarcoma	89	[13]
16H5	Rheumatoid arthritis	50	[14]
17-1A	Colorectal CA	100	[15]
A7-NCS	Colorectal carcinoma, pancreatic carcinoma	100	[16]
Anti-CEA antibody fragments (A5B7-F(ab′)2), conjugated to bacterial enzyme, carboxypeptidase G2 (CPG2) followed by a galactosylated anti-CPG2 mAb (SB43)	CEA-bearing tumors	100	[17]
Anti-CEA, anti-In-DTPA bispecific Fab′–Fab	Colorectal carcinoma (imaging)	64	[18]
Anti-melanoma or anti-CEA mAbs	Melanoma, colorectal carcinoma (imaging)	83	[19]
Arcitumomab	Colorectal carcinoma for imaging	0	[20]
B43-Genistein	B-lineage acute lymphoblastic leukemia	33	[21]
B72.3	Colorectal carcinoma	44% with 111In-SCN-Bz-diethylenetriaminepenta-acetic acid (DTPA)-labeled. 26% with In-111 (In-111) B72.3 glycyl-tyrosyl-*n*-diethylenetriaminepenta-acetic acid lysine (GYK-DTPA)-labeled	[22,23]
B-C7	Septic shock	100	[24]
B-E8	Myeloma, renal cell carcinoma	75	[25]
BrE-3, 111In-MX-DTPA	Human ductal breast cancer	83	[26]
BW 250/18399mTc-labeled	Detection of inflammatory lesions (imaging)	1	[27]
BW 431/2699mTc-labeled	Colorectal and lung adenocarcinoma (imaging)	33	[28]
BW 494/BI 51.011	Pancreatic cancer	94	[29]
CCR086 indium-111-labeled	Detection of colorectal carcinoma metastases (imaging)	80	[30]
CYT-103 111In-labeled anti-TAG-72 mAb	Colorectal carcinoma (imaging)	42	[31]
D612	Metastatic gastrointestinal cancer	86	[32]
E5/Edobacomab	Gram negative sepsis	20	[33]
HMFG1, HMFG2, H17E2, B72.3	Ovarian cancer radioimmunotherapy	100	[34]
HRS-3/A9	Hodgkin's disease	46	[35]
Ibritumomab-Tiuxetan/Zevalin	Non-Hodgkin's lymphoma	<2	[36]
IgM mAbs,WM63(CD48)and WM66	Chronic lymphocytic leukemia	0	[37]
Ior EGF/r3	Gliomas or meningiomas	89	[38]
LL2	Non-Hodgkins lymphoma	19	[39]
L6	Adenocarcinoma	64	[40]
M195	Myeloid leukemias, myelodysplastic syndromes	37	[41]
MAb with high human immunodeficiency virus type 1 (HIV-1) neutralizing titers	Human immunodeficiency virus	73	[42]
OC/TR bispecific mAb	Intraperitoneal (i.p.)treatment of ovarian cancer	100	[43]
OC125	Ovarian cancers	100	[44]
OKB7 I131-labeled	CD21-positive, non-Hodgkin's lymphoma	75	[45]
OKT3	Graft rejection	86	[46]
OV-TL 3	Ovarian cancer	40	[47]
T101	Cutaneous T-cell lymphoma or chronic lymphocytic leukemia	100	[12]
I131-tositumomab/Bexxar	Non-Hodgkin's lymphoma	8	[48]
YTH 24.5 and YTH 54.12	Pretreatment of donor organs for transplantation	5	[49]
ZCE 025	Colorectal carcinoma	64/up to 100% with 111In-ZCE 025-labeled form	[50]
ZME 018 and 96.5	Melanoma or basal cell carcinoma	88	[51]

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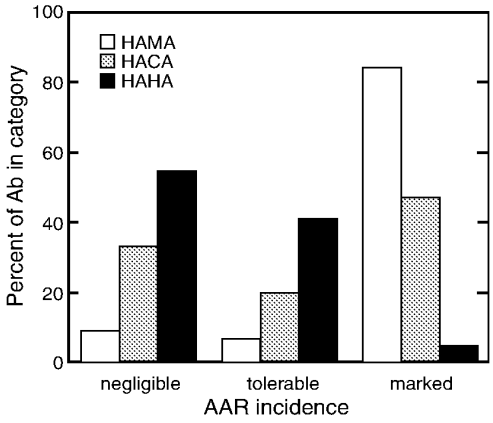


Fig. 1. AAR incidence compared to molecular format. Antibody and immunogenicity incidence data pairs from each clinical study were sorted according to molecular format (mouse, chimeric, and humanized) and binned according to immunogenicity level (negligible, tolerable, and marked) as defined in the text. Bars in the histogram mark the percentage of antibodies, of those of a particular format, whose immunogenicity falls within the indicated level.

common than chimeric mAb in this operational category. However, if the marked category is discounted, humanized and chimeric mAb both have about equal proportions of *tolerable* and *negligible* examples (Fig. 1).

3.4. Does immunosuppression reduce AAR?

Very few studies report controlled comparisons between AAR with concomitant immunosuppression and without. Humira (adalimumab), a phage-derived human anti-TNFα antibody, was immunogenic in 12% of patients, whereas a cohort treated additionally with methotrexate showed 1% AAR [3]. Remicade (infliximab), a chimeric antibody to the same antigen, dosed repeatedly gave AAR in 75% of patients treated with mAb alone, versus 43% of patients concurrently taking immunosuppressive drugs [4]. In a study of Humicade, a humanized anti-TNFα, concomitant use of immune modifiers was incorporated into a complex study design, and was not observed to affect the incidence of AAR [5]. Simulect (basiliximab), a chimeric anti-CD25, gave a contrasting result: 1.4% of transplant patients showed HACA, but this incidence jumped to 12% among patients who also received the murine anti-CD3 antibody muronomab-CD3. In this case, the mouse antibody, though immunosuppressive, may potentiate latent reactivity with murine structures on the chimeric antibody. Immunosuppression of HACA or HAHA, though in keeping with immunological theory, is not necessarily a certainty, and indeed formally, the exceptions are as numerous as the positive examples.

3.5. Is AAR less with anti-B-lymphocyte antibodies?

Since anti-B-cell antibodies would be expected to attenuate the cells responsible for the humoral AAR, immunological theory predicts that mAb to B-cell markers would be inherently less immunogenic than other therapeutic mAb. Clinical immunogenicity data support this hypothesis. Among chimeric and humanized antibodies, evidence is anecdotal. Rituximab and Campath-

Table 2
Reported human anti-chimeric antibody (HACA) responses

Antibody/synonyms	Antigen; indication	% of patients with AAR	References
Rituximab/Rituxan	CD20; B-cell non-Hodgkin's lymphoma	0	[52]
Abciximab	β3integrin of the GPIIb/IIIa and $\alpha_v\beta_3$ receptors on human platelets; adjunct to percutaneous transluminal coronary angioplasty or atherectomy (PCTA) for the prevention of acute cardiac ischemic complications	4.8. After a first readministration, an additional 19.0% became HACA-positive	[53]
Basiliximab/Simulect	CD25; prophylaxis/treatment of organ/hematopoietic stem cell transplant or graft versus host disease	11.8% with OKT3, 1.4% without OKT3	[54]
Infliximab/Remicade	TNFα; Crohn's disease, rheumatoid arthritis	61	[4]
111In-labeled c-Nd2	Pancreatic cancer (imaging)	0	[55]
131I-labeled chimeric L6	Breast cancer	67%. None if concomitant cyclosporin	[56]
cG250	Clear cell renal cancer	0%. 12.5% with I-131-labeled Ab	[57,58]
ch14.18	Ganglioside GD 2; neuroblastoma	0	[59]
chA7Fab	CEA expressing tumors	71	[60]
Chimeric OC/TR	Bispecific F(ab′)2 against CD3 and human folate-binding protein (FBP) on non-mucinous ovarian carcinomas; ovarian carcinoma	50	[61]
cMOv18	Ovarian carcinoma	0	[62,63]
cM-T412	CD4; rheumatoid arthritis	60	[64]
Anti-CEA mAb/cT84.66	Metastatic CEA-producing malignancies	7	[65]
(186)Re-labeled chimeric U36	CD44v6; head and neck cancer	40	[66]
Cetuximab/Erbitux	Epidermal growth factor receptor; colorectal cancer	5	[67]

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Table 3
Reported human anti-humanized antibody (HAHA) responses

Antibody/synonyms	Antigen; indication	% of patients with AAR	References
Alemtuzumab/Campath-1H	CD52; B-cell chronic lymphocytic leukemia (B-CLL)	1.9	[68]
Daclizumab/Zenapax	CD25; prophylaxis of acute organ rejection in patients receiving renal transplants, to be used as part of an immunosuppressive regimen that includes cyclosporine and corticosteroids	14% low titer anti-idiotype Ab, 34% in pediatrics	[69]
Palivizumab/Synagis	Respiratory syncytial virus; prophylaxis in pediatric patients at high risk of RSV disease	1	[70]
Trastuzumab/Herceptin	HER2/neu (human epidermal growth factor 2); metastatic breast cancer with tumors overexpressing the HER2 protein	0.1	[71]
16.88/CTA 16.88	CTA 16.88-bearing epithelial-derived tumors including carcinomas of the colon, pancreas, breast, ovary, and lung	0	[72]
88BV59/CTA 16.88	CTA 16.88-bearing epithelial-derived tumors (different epitope)	3.7% low titer after a single administration of 88BV59. 0.3% with Tc99m version	[72,73]
hu-A33	"A33" colonic epithelium Ag; colon cancer	Early onset (49%) peaked at 2 weeks, then declined. Later onset (17%) continued building	[2]
Bivatuzumab/BIWA 4	CD44v6; head and neck squamous cell carcinoma (HNSCC)	0% with Tc-99m version. 10% with (186)Re-labeled version	[74,75]
Humicade/CDP571	TNFα; Crohn's disease	7	[5]
Gemtuzumab Ozogamicin/ Mylotarg	CD33; acute myeloid leukemia	2.9	[76]
Hu23F2G	CD11/CD18; acute non-infectious inflammatory disorders mediated predominantly by neutrophils, e.g., multiple sclerosis	0	[77]
Hu2PLAP	Placental alkaline phosphatase	0	[78]
Hu5c8	CD154 (CD40 ligand); hemophilia (by suppressing anti-factor VIII antibodies)	0	[79]
HuBrE-3	Breast cancer	14	[80]
HuM291	CD3; renal transplant and graft versus host disease	0	[81]
Adalimumab/Humira	TNFα; developed by phage display of a human antibody library	5% low titer neutralizing antibodies. 1% with methotrexate, versus 12% without	[3]
Natalizumab	α4 integrin; Crohn's disease	7	[82]
Omalizumab	Allergic asthma	0	[83]
Efalizumab/Raptiva	CD11a; psoriasis	6.3	[84]
Bevacizumab/Avastin, rhu anti-VEGF mAb	VEGF expressing solid tumors	0	[85,86]
Cantuzumab Mertansine	CanAg glycoform of MUC1; colorectal cancer; humanized by "resurfacing" technology	0	[87]
Vitaxin	integrin $\alpha_v\beta_3$ (vitronectin receptor); metastatic cancer	0	[88,89]

1H give 0 and 2% AAR, respectively, in the lower echelon of incidence data. Among anti-B-cell mouse mAb, the median incidence is 34%, whereas the median incidence among mAb with non-B-cell targets is 75%. The biological activity of anti-B-lymphocyte mAb thus may yield a low AAR that masks their intrinsic antigenicity.

4. Discussion

4.1. Immunogenicity vs. molecular format

The data tables and histogram in Fig. 1 make clear that the largest step in reducing immunogenicity of therapeutic mAb comes from replacement of murine constant regions with human ones. Humanization of variable regions appears to decrease immunogenicity further. However, the overlap between HAHA and HACA incidence is extensive and blurs the advantage of humanization. Nevertheless, the frequency of marked HACA and the infrequency of marked HAHA suggest that a choice not to humanize entails a ponderable business risk. A parallel question, whether "fully human" mAb are less immunogenic than humanized mAb cannot be answered at present, because full immunogenicity data are available for just one mAb developed from phage-displayed human libraries. Repetitively dosed Humira showed AAR in 12% of patients—at the higher end of AAR incidence of the humanized antibodies listed in Table 3 [3].

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4.2. Toward standardization of immunogenicity analysis

In the evaluation of the immunogenicity of humanized, chimeric, and human antibodies, we encounter a lack of a rational quantitative scale for immunogenicity, inability to compare relative immunogenicity between molecules, incomplete clinical data on immunogenicity, and failure to relate immunogenicity to molecular structure/properties such as mutational differences away from germline and anti-allotype reactivity. Emerging technologies and trends may repair this situation.

Biosensor technology provides a means of converting serum reactivity to an absolute scale of the anti-antibody content of individual sera [2]. The traditional method of analyzing antigen binding by polyclonal antisera is to fit binding data to a Sips distribution [6]. A Sips analysis gives the quantity of specific antibody in a sample, its average avidity, and spread of avidity constant. This approach has not been applied to AAR, but its application could facilitate inter-study comparisons.

Anti-allotype reactions are predicted to occur during therapy of a genetically diverse population with a single antibody reagent, but with some exceptions [7], anti-allotype AAR has not been measured. Anti-allotype AAR may be of future significance because in principle it can be circumvented by "personalized" medicine in which a patient receives an allotype-matched therapeutic mAb.

Lastly, the recent announcement by a major pharmaceutical manufacturer that it will publicize the results of all clinical trials of marketed products—both unsuccessful and successful—may portend a similar trend throughout the industry. Reporting of failed trials of therapeutic antibodies may well alter a future version of the histogram in Fig. 1.

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ICH Topic Q 5 C
Quality of Biotechnological Products:
Stability Testing of Biotechnological/Biological Products

Step 5

NOTE FOR GUIDANCE ON QUALITY OF BIOTECHNOLO-GICAL PRODUCTS: STABILITY TESTING OF BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS
(CPMP/ICH/138/95)

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QUALITY OF BIOTECHNOLOGICAL PRODUCTS:
STABILITY TESTING OF BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS

Annex to the ICH Harmonised Tripartite Guideline for the Stability Testing of New Drug Substances and Products

1. PREAMBLE

The guidance stated in the ICH Harmonised Tripartite Guideline 'Stability Testing of New Drug Substances and Products' (27 October 1993) applies in general to biotechnological/biological products. However, biotechnological/biological products do have distinguishing characteristics to which consideration should be given in any well-defined testing program designed to confirm their stability during the intended storage period. For such products, in which the active components are typically proteins and/or polypeptides, maintenance of molecular conformation and, hence of biological activity, is dependent on noncovalent as well as covalent forces. The products are particularly sensitive to environmental factors such as temperature changes, oxidation, light, ionic content, and shear. In order to ensure maintenance of biological activity and to avoid degradation, stringent conditions for their storage are usually necessary.

The evaluation of stability may necessitate complex analytical methodologies. Assays for biological activity, where applicable, should be part of the pivotal stability studies. Appropriate physicochemical, biochemical and immunochemical methods for the analysis of the molecular entity and the quantitative detection of degradation products should also be part of the stability program whenever purity and molecular characteristics of the product permit use of these methodologies.

With the above concerns in mind, the applicant should develop the proper supporting stability data for a biotechnological/biological product and consider many external conditions which can affect the product's potency, purity and quality. Primary data to support a requested storage period for either drug substance or drug product should always be based on long-term, real-time, real-condition stability studies. Thus, the development of a proper long-term stability program becomes critical to the successful development of a commercial product. The purpose of this document is to give guidance to applicants regarding the type of stability studies that should be provided in support of marketing applications. It is understood that during the review and evaluation process, continuing updates of initial stability data may occur.

2. SCOPE OF THE ANNEX

The guidance stated in this annex applies to well-characterised proteins and polypeptides, their derivatives and products of which they are components, and which are isolated from tissues, body fluids, cell cultures, or produced using rDNA technology. Thus, the document covers the generation and submission of stability data for products such as cytokines (interferons, interleukins, colony-stimulating factors, tumour necrosis factors), erythropoietins, plasminogen activators, blood plasma factors, growth hormones and growth factors, insulins, monoclonal antibodies, and vaccines consisting of well-characterised proteins or polypeptides. In addition, the guidance outlined in the following sections may apply to other types of products, such as conventional vaccines, after consultation with the appropriate regulatory authorities. The document does not cover antibiotics, allergenic extracts, heparins, vitamins or whole blood.

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3. TERMINOLOGY

For the basic terms used in this annex the reader is referred to the "Glossary" in the ICH Harmonised Tripartite Guideline 'Stability Testing of New Drug Substances and Products' (27 October 1993). However, since manufacturers of biotechnological/biological products sometimes use traditional terminology, traditional terms are specified in parentheses to assist the reader. A supplemental glossary is also included that explains certain terms used in the production of biotechnological/biological products.

4. SELECTION OF BATCHES

4.1 Drug Substance (Bulk Material)

Where bulk material is to be stored after manufacture but prior to formulation and final manufacturing, stability data should be provided on at least three batches for which manufacture and storage are representative of the manufacturing scale of production. A minimum of six months stability data at the time of submission should be submitted in cases where storage periods greater than six months are requested. For drug substances with storage periods of less than six months, the minimum amount of stability data in the initial submission should be determined on a case-by-case basis. Data from pilot-plant-scale batches of drug substance produced at a reduced scale of fermentation and purification may be provided at the time the dossier is submitted to the regulatory agencies with a commitment to place the first three manufacturing scale batches into the long-term stability program after approval.

The quality of the batches of drug substance placed into the stability program should be representative of the quality of the material used in pre-clinical and clinical studies and of the quality of the material to be made at manufacturing scale. In addition, the drug substance (bulk material) made at pilot-plant scale should be produced by a process and stored under conditions representative of that used for the manufacturing scale. The drug substance entered into the stability program should be stored in containers which properly represent the actual holding containers used during manufacture. Containers of reduced size may be acceptable for drug substance stability testing provided that they are constructed of the same material and use the same type of container/closure system that is intended to be used during manufacture.

4.2 Intermediates

During manufacture of biotechnological/biological products, the quality and control of certain intermediates may be critical to the production of the final product. In general, the manufacturer should identify intermediates and generate in-house data and process limits that assure their stability within the bounds of the developed process. While the use of pilot-plant-scale data is permissible, the manufacturer should establish the suitability of such data using the manufacturing-scale process.

4.3 Drug Product (Final Container Product)

Stability information should be provided on at least three batches of final container product representative of that which will be used at manufacturing scale. Where possible, batches of final container product included in stability testing should be derived from different batches of bulk material. A minimum of six months data at the time of submission should be submitted in cases where storage periods greater than six months are requested. For drug products with storage periods of less than six months, the minimum amount of stability data in the initial submission should be determined on a case-by-case basis. Product expiration dating will be based upon the actual data submitted in support of the application. Since dating is based upon the real-time/real-temperature data submitted for review, continuing updates of initial stability data should occur during the review and evaluation process. The quality of the final container

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product placed on stability studies should be representative of the quality of the material used in the preclinical and clinical studies. Data from pilot-plant scale batches of drug product may be provided at the time the dossier is submitted to the regulatory agencies with a commitment to place the first three manufacturing scale batches into the long-term stability program after approval. Where pilot-plant scale batches were submitted to establish the dating for a product and, in the event that product produced at manufacturing scale does not meet those long-term stability specifications throughout the dating period or is not representative of the material used in pre-clinical and clinical studies, the applicant should notify the appropriate regulatory authorities to determine a suitable course of action.

4.4 Sample selection criteria

Where one product is distributed in batches differing in fill volume (e.g., 1 milliliter (ml), 2 ml, or 10 ml), unitage (e.g., 10 units, 20 units, or 50 units), or mass (e.g., 1 milligram (mg), 2 mg, or 5 mg) samples to be entered into the stability program may be selected on the basis of a matrix system and/or by bracketing.

Matrixing, i.e., the statistical design of a stability study in which different fractions of samples are tested at different sampling points, should only be applied when appropriate documentation is provided that confirms that the stability of the samples tested represents the stability of all samples. The differences in the samples for the same drug product should be identified as, for example, covering different batches, different strengths, different sizes of the same closure and possibly, in some cases, different container/closure systems. Matrixing should not be applied to samples with differences that may affect stability, such as different strengths and different containers/closures, where it cannot be confirmed that the products respond similarly under storage conditions.

Where the same strength and exact container/closure system is used for three or more fill contents, the manufacturer may elect to place only the smallest and largest container size into the stability program, i.e., bracketing. The design of a protocol that incorporates bracketing assumes that the stability of the intermediate condition samples are represented by those at the extremes. In certain cases, data may be needed to demonstrate that all samples are properly represented by data collected for the extremes.

5. STABILITY-INDICATING PROFILE

On the whole, there is no single stability-indicating assay or parameter that profiles the stability characteristics of a biotechnological/biological product. Consequently, the manufacturer should propose a stability-indicating profile that provides assurance that changes in the identity, purity and potency of the product will be detected.

At the time of submission, applicants should have validated the methods that comprise the stability-indicating profile and the data should be available for review. The determination of which tests should be included will be product-specific. The items emphasised in the following subsections are not intended to be all-inclusive, but represent product characteristics that should typically be documented to adequately demonstrate product stability.

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5.1 Protocol

The dossier accompanying the application for marketing authorisation should include a detailed protocol for the assessment of the stability of both drug substance and drug product in support of the proposed storage conditions and expiration dating periods. The protocol should include all necessary information which demonstrates the stability of the biotechnological/biological product throughout the proposed expiration dating period including, for example, well-defined specifications and test intervals. The statistical methods that should be used are described in the Tripartite Guideline on stability.

5.2 Potency

When the intended use of a product is linked to a definable and measurable biological activity, testing for potency should be part of the stability studies. For the purpose of stability testing of the products described in this guideline, potency is the specific ability or capacity of a product to achieve its intended effect. It is based on the measurement of some attribute of the product and is determined by a suitable quantitative method. In general, potencies of biotechnological/biological products tested by different laboratories can be compared in a meaningful way only if expressed in relation to that of an appropriate reference material. For that purpose, a reference material calibrated directly or indirectly against the corresponding national or international reference material should be included in the assay.

Potency studies should be performed at appropriate intervals as defined in the stability protocol and the results should be reported in units of biological activity calibrated, whenever possible, against nationally or internationally recognised standard. Where no national or international standards exists, the assay results may be reported in in-house derived units using a characterised reference material.

In some biotechnological/biological products, potency is dependent upon the conjugation of the active ingredient(s) to a second moiety or binding to an adjuvant. Dissociation of the active ingredient(s) from the carrier used in conjugates or adjuvants should be examined in real-time/real-temperature studies (including conditions encountered during shipment). The assessment of the stability of such products may be difficult since, in some cases, in vitro tests for biological activity and physicochemical characterisation are impractical or provide inaccurate results. Appropriate strategies (e.g., testing the product prior to conjugation/binding, assessing the release of the active compound from the second moiety, in vivo assays) or the use of an appropriate surrogate test should be considered to overcome the inadequacies of in vitro testing.

5.3 Purity and Molecular Characterisation

For the purpose of stability testing of the products described in this guideline, purity is a relative term. Due to the effect of glycosylation, deamidation, or other heterogeneities, the absolute purity of a biotechnological/biosological product is extremely difficult to determine. Thus, the purity of a biotechnological/biological product should be typically assessed by more than one method and the purity value derived is method-dependent. For the purpose of stability testing, tests for purity should focus on methods for determination of degradation products.

The degree of purity, as well as individual and total amounts of degradation products of the biotechnological/biological product entered into the stability studies, should be reported and documented whenever possible. Limits of acceptable degradation should be derived from the analytical profiles of batches of the drug substance and drug product used in the pre-clinical and clinical studies.

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The use of relevant physicochemical, biochemical and immunochemical analytical methodologies should permit a comprehensive characterisation of the drug substance and/or drug product (e.g., molecular size, charge, hydrophobicity) and the accurate detection of degradation changes that may result from deamidation, oxidation, sulfoxidation, aggregation or fragmentation during storage. As examples, methods that may contribute to this include electrophoresis (SDS-PAGE, immunoelectrophoresis, Western blot, isoelectrofocusing), high-resolution chromatography (e.g., reversed-phase chromatography, gel filtration, ion exchange, affinity chromatography), and peptide mapping.

Wherever significant qualitative or quantitative changes indicative of degradation product formation are detected during long-term, accelerated and/or stress stability studies, consideration should be given to potential hazards and to the need for characterisation and quantification of degradation products within the long-term stability program. Acceptable limits should be proposed and justified, taking into account the levels observed in material used in pre-clinical and clinical studies.

For substances that can not be properly characterised or products for which an exact analysis of the purity cannot be meaningfully determined through routine analytical methods, the applicant should propose and justify alternative testing procedures.

5.4 Other Product Characteristics

The following product characteristics, though not specifically relating to biotechnological/biological products, should be monitored and reported for the drug product in its final container:

- Visual appearance of the product (colour and opacity for solutions/suspensions; colour, texture and dissolution time for powders), visible particulates in solutions or after the reconstitution of powders or lyophilised cakes, pH, and moisture level of powders and lyophilised products.

- Sterility testing or alternatives (e.g., container/closure integrity testing) should be performed at a minimum initially and at the end of the proposed shelf-life.

- Additives (e.g., stabilisers, preservatives) or excipients may degrade during the dating period of the drug product. If there is any indication during preliminary stability studies that reaction or degradation of such materials adversely affect the quality of the drug product, these items may need to be monitored during the stability program.

- The container/closure has the potential to adversely affect the product and should be carefully evaluated (see below).

6. STORAGE CONDITIONS

6.1 Temperature

Since most finished biotechnological/biological products need precisely defined storage temperatures, the storage conditions for the real-time/real-temperature stability studies may be confined to the proposed storage temperature.

6.2 Humidity

Biotechnological/biological products are generally distributed in containers protecting them against humidity. Therefore, where it can be demonstrated that the proposed containers (and

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conditions of storage) afford sufficient protection against high and low humidity, stability tests at different relative humidities can usually be omitted. Where humidity-protecting containers are not used, appropriate stability data should be provided.

6.3 Accelerated and stress conditions

As previously noted, the expiration dating should be based on real-time/real-temperature data. However, it is strongly suggested that studies be conducted on the drug substance and drug product under accelerated and stress conditions. Studies under accelerated conditions may provide useful support data for establishing the expiration date, provide product stability information for future product development (e.g., preliminary assessment of proposed manufacturing changes such as change in formulation, scale-up), assist in validation of analytical methods for the stability program, or generate information which may help elucidate the degradation profile of the drug substance or drug product. Studies under stress conditions may be useful in determining whether accidental exposures to conditions other than those proposed (e.g., during transportation) are deleterious to the product and also for evaluating which specific test parameters may be the best indicators of product stability. Studies of the exposure of the drug substance or drug product to extreme conditions may help to reveal patterns of degradation; if so, such changes should be monitored under proposed storage conditions. While the Tripartite Guideline on stability describes the conditions of the accelerated and stress study, the applicant should note that those conditions may not be appropriate for biotechnological/biological products. Conditions should be carefully selected on a case-by-case basis.

6.4 Light

Applicants should consult the appropriate regulatory authorities on a case-by-case basis to determine guidance for testing.

6.5 Container/Closure

Changes in the quality of the product may occur due to the interactions between the formulated biotechnological/biological product and container/closure. Where the lack of interactions cannot be excluded in liquid products (other than sealed ampoules), stability studies should include samples maintained in the inverted or horizontal position (i.e., in contact with the closure), as well as in the upright position, to determine the effects of the closure on product quality. Data should be supplied for all different container/closure combinations that will be marketed.

In addition to the standard data necessary for a conventional single-use vial, the applicant should demonstrate that the closure used with a multiple-dose vial is capable of withstanding the conditions of repeated insertions and withdrawals so that the product retains its full potency, purity, and quality for the maximum period specified in the instructions-for-use on containers, packages, and/or package inserts. Such labelling should be in accordance with relevant national/regional requirements.

6.6 Stability after Reconstitution of Freeze-Dried Product

The stability of freeze-dried products after their reconstitution should be demonstrated for the conditions and the maximum storage period specified on containers, packages, and/or package inserts. Such labelling should be in accordance with relevant national/regional requirements.

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7. TESTING FREQUENCY

The shelf-lives of biotechnological/biological products may vary from days to several years. Thus, it is difficult to draft uniform guidelines regarding the stability study duration and testing frequency that would be applicable to all types of biotechnological/biological products. With only a few exceptions, however, the shelf-lives for existing products and potential future products will be within the range of 0.5 to five years. Therefore, the guidance is based upon expected shelf-lives in that range. This takes into account the fact that degradation of biotechnological/biological products may not be governed by the same factors during different intervals of a long storage period.

When shelf-lives of one year or less are proposed, the real-time stability studies should be conducted monthly for the first three months and at three-month intervals thereafter.

For products with proposed shelf-lives of greater than one year, the studies should be conducted every three months during the first year of storage, every six months during the second year, and annually thereafter.

While the testing intervals listed above may be appropriate in the pre-approval or pre-license stage, reduced testing may be appropriate after approval or licensure where data are available that demonstrate adequate stability. Where data exist that indicate the stability of a product is not compromised, the applicant is encouraged to submit a protocol which supports elimination of specific test intervals (e.g., nine-month testing) for post-approval/post-licensure, long-term studies.

8. SPECIFICATIONS

Although biotechnological/biological products may be subject to significant losses of activity, physicochemical changes, or degradation during storage, international and national regulations have provided little guidance with respect to distinct release and end of shelf-life specifications. Recommendations for maximum acceptable losses of activity, limits for physicochemical changes, or degradation during the proposed shelf-life have not been developed for individual types or groups of biotechnological/biological products but are considered on a case-by-case basis. Each product should retain its specifications within established limits for safety, purity, and potency throughout its proposed shelf-life. These specifications and limits should be derived from all available information using the appropriate statistical methods. The use of different specifications for release and expiration should be supported by sufficient data to demonstrate that clinical performance is not affected as discussed in the Tripartite Guideline on stability.

9. LABELLING

For most biotechnological/biological drug substances and drug products, precisely defined storage temperatures are recommended. Specific recommendations should be stated, particularly for drug substances and drug products that cannot tolerate freezing. These conditions, and where appropriate, recommendations for protection against light and/or humidity, should appear on containers, packages, and/or package inserts. Such labelling should be in accordance with relevant national regional requirements.

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GLOSSARY

Conjugated Product

A conjugated product is made up of an active ingredient (for example, peptide, carbohydrate) bound covalently or noncovalently to a carrier (for example, protein, peptide, inorganic mineral) with the objective of improving the efficacy or stability of the product.

Degradation Product

A molecule resulting from a change in the drug substance (bulk material) brought about over time. For the purpose of stability testing of the products described in this guideline, such changes could occur as a result of processing or storage (e.g., by deamidation, oxidation, aggregation, proteolysis). For biotechnological/biological products some degradation products may be active.

Impurity

Any component of the drug substance (bulk material) or drug product (final container product) which is not the chemical entity defined as the drug substance, an excipient, or other additives to the drug product.

Intermediate

For biotechnological/biological products, a material produced during a manufacturing process which is not the drug substance or the drug product but whose manufacture is critical to the successful production of the drug substance or the drug product. Generally, an intermediate will be quantifiable and specifications will be established to determine the successful completion of the manufacturing step prior to continuation of the manufacturing process. This includes material which may undergo further molecular modification or be held for an extended period of time prior to further processing.

Manufacturing-Scale Production

Manufacture at the scale typically encountered in a facility intended for product production for marketing.

Pilot-Plant Scale

The production of the drug substance or drug product by a procedure fully representative of and simulating that to be applied at manufacturing scale. The methods of cell expansion, harvest, and product purification should be identical except for the scale of production.

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Ig Constant Region Effects on Variable Region Structure and Function

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The adaptive humoral immune response is responsible for the generation of antimicrobial proteins known as immunoglobulin molecules or antibodies. Immunoglobulins provide a defense system against pathogenic microbes and toxins by targeting them for removal and/or destruction. Historically, antibodies have been thought to be composed of distinct structural domains known as the variable and constant regions that are responsible for antigen binding and mediating effector functions such as opsonization and complement activation, respectively. These domains were thought to be structurally and functionally independent. Recent work has revealed however, that in some families of antibodies, the two regions can influence each other. We will discuss the body of work that led to these observations, as well as the mechanisms that have been proposed to explain how these two different antibody regions may interact in the function of antigen binding.

Keywords: immunoglobulin, isotype, constant region, variable region, structure, function

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INTRODUCTION

Antibodies (Abs), or immunoglobulin (Ig) molecules are antimicrobial proteins that are secreted by B lymphocytes, and serve as critical participants in the adaptive immune response. The main function of Abs is to bind foreign molecules in the serum and other bodily fluids and, in most cases, label them for removal. This occurs through a form of molecular guilt by association, involving non-covalent binding of Abs to their antigens (Ag) and to cellular Fc receptors (FcRs). This removal is mediated by a variety of mechanisms associated with Ab function such as facilitation of phagocytosis, complement activation, and Ab dependent cellular cytotoxicity.

The Ig molecule consists of two polypeptide chains, a heavy (H) and a light (L) chain, each of which is composed of two regions, a constant region (C) and a variable region (V) (**Figure 1**). These chains form monomers which then combine into dimers, or higher-order oligomers to form a full Ig molecule. Both C and V regions contain domains from the H and L chains (Dreyer and Bennett, 1965). Functionally, the CH region confers effector properties such as complement binding, half-life length, interactions with FcRs, and the class, or isotype, of the Ig. In both humans and mice, there are four IgG, or γ-chain isotypes that are important for the identification and clearance of many peptide and polysaccharide Ags (Tonegawa, 1983). In contrast, the V region confers specificity to the Ig molecule by functioning as the direct contact between the Ig and its Ags.

Abbreviations: Ig, immunoglobulin; Ab, antibody; Ag, antigen; mAb, monoclonal antibody; CH, heavy chain constant region; CL, light chain constant region; Fab, antigen binding fragment; Fc, crystallizable fragment; SPR, Surface Plasmon Resonance; ITC, Isothermal Calorimetry; ELISA, Enzyme-Linked Immunosorbent Assay.

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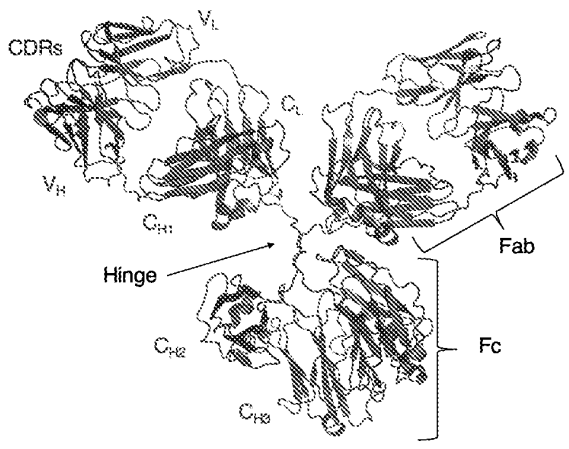


FIGURE 1 | Crystal structure of intact IgG_1 (PDB ID: 1IGY). Adapted from Harris et al. (1998).

BACKGROUND

Since the mid-twentieth century, the Ig molecule has been considered a bifunctional molecule consisting of two largely independent regions, a V region responsible for specificity and affinity, and a C region responsible for effector functions such as complement activation and interaction with FcRs. This view emerged from biochemical studies in the late 1950s, when Nobel laureate Rodney R. Porter used proteolytic digestion to cleave Ab molecules into fragments. These fragments eventually became known as the Ag binding fragment, or Fab, and the Fc fragment, because it could be easily crystallized (Porter, 1957; Porter and Press, 1957). One decade later, as the protein crystallography field progressed, X-ray studies of Fab fragments provided structural evidence consistent with the presumed functional separation of each adjacent V and C domain on both the H and L chain. For example, V_L domains were found to be separated from their C_{H1} domain neighbors by long, disordered polypeptide chains that were interpreted as spacers both physically and functionally (Davies et al., 1975). This observation was confirmed by additional studies in the 1990's (Harris et al., 1997). The disordered nature of the spacers was interpreted as inconsistent with a tight structural connection where the C influenced the structure of the V and vice versa.

STRUCTURAL AND BIOCHEMICAL EVIDENCE THAT SUGGESTS TWO INDEPENDENT DOMAINS

The early hypotheses that the Fab region acted independently from the whole Ig were based on X-ray crystallographic studies of Fab and Fc fragments, Fab-hapten studies, and electron microscopy (EM) analysis of Ab–Ag complexes. Hapten studies provided additional support for the notion of two independent Ig regions as no evidence was found for structural changes in Fab molecules upon hapten binding (Stryer and Griffith, 1965; Stryer, 1968; Yguerabide et al., 1970; Werner et al., 1972; Segal et al., 1974; Harris et al., 1997). EM studies also failed to show differences in Fab tertiary structure whether this region was bound to Ag alone or as part of a whole IgG (Feinstein and Rowe, 1965).

Furthermore, the presence of hypervariable regions, or complementarity determining regions (CDRs) in the V and not the C domains suggested the evolutionary importance of the specific conformation of each V region in Ag binding (Stryer and Griffith, 1965; Stryer, 1968; Yguerabide et al., 1970; Werner et al., 1972; Segal et al., 1974; Kabat et al., 1977; Chothia et al., 1989; Harris et al., 1997). Then, in the late 1970s, Tonegawa and collaborators showed that distinct genes encoding V and C regions were rearranged and combined to express the Ig molecule, providing a mechanism for the generation of Ab diversity while maintaining Ig effector function (Tonegawa, 1983). While V regions were shown to diversify during their adaptation to specific Ags, C regions maintained their structure, supporting the hypothesis that these two regions were functionally independent.

In the early 1980s, fluorescence studies by Oi et al. failed to show an interaction between Ig V and C regions upon Ag binding (Oi et al., 1984). This negative evidence was nevertheless fully consistent with the evolving view of two independent domains. More recently, X-ray crystallographic studies of intact Igs have revealed a highly flexible hinge region described as a "loose tether" separating the Fab and Fc regions, allowing them to move freely with respect to one another (Saphire et al., 2002). We note that the classical view of two independent Ig domains emerged largely from the interpretation of negative data from a variety of structural techniques. However, there was also a certain elegance to the notion of two domains, one focused on binding and the other on downstream function, which appealed to the reductionist tenor of the times.

EARLY CHALLENGES TO THE MODEL

Ab–Ag complexes are readily ingested by FcR-bearing cells in contrast to the minimal ingestion of free Ab. This observation raised the possibility that the greater affinity of Ab–Ag complexes for FcRs was the result of a structural change in the Ab molecule upon binding Ag. In 1976, Huber et al. proposed an allosteric Ig model whereby Ag binding triggered a signal that traveled from the V to the C regions (Huber et al., 1976). This signal was thought to run from the V domain along conserved residues involved in inter-chain and inter-domain contacts to reach the C domain and cause a structural change that could enhance affinity for FcRs. In their final model, based on X-ray crystallographic data as well as molecular dynamics simulations of a full IgG molecule, they emphasized that all longitudinal contacts were closed. They stated that this resulted in a rigid structure where Fab_2 bending was inhibited by C_{H1}–C_{H2} interactions. They further suggested that Ag binding caused overall Ig stiffening. This stiffening was thought to occur because the flexible hinge region allowed for their hypothesized C_{H1}–C_{H2} contacts, resulting in a rigid, T-shaped, Ig (Huber et al.,

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1976). These inferences suggested that C_{H1}–C_{H2} contacts exist and are critical to Ag binding. They based their modeling studies of X-ray crystallographic IgG data on the hypotheses that Igs had both segmental rigidity and overall flexibility, and that free Fab molecules were rigid (Huber et al., 1976). These inferences were in turn based on the earlier Fab-hapten binding studies (Stryer and Griffith, 1965; Stryer, 1968; Yguerabide et al., 1970; Werner et al., 1972; Segal et al., 1974).

During this time it was also suggested that Ag binding may result in Ab conformational changes that lead to complex formation, which are in turn readily identified by macrophage FcR for uptake (Shinomiya and Koyama, 1976). This view was based on older evidence that Ab–Ag complexes were cleared from the serum at much faster rates than free Ab (Benacerraf et al., 1959). However, the view of an allosteric change in the Ab molecule that facilitated the interaction with FcR did not gain favor and was supplanted by the explanation that the greater affinity of Ag–Ab complexes was due to cross-linking of FcRs. Again, the allosteric view was abandoned largely due to negative data, while cross-linking of FcRs was a demonstrable effect that triggered signal transduction and explained increased binding of Ag–Ab complexes on the basis of increased avidity without the necessity of conformational changes.

By the 1990's the notion that Ig molecules comprised two independent non-interacting domains had largely taken root and become immunological dogma. However, several studies reported observations that seemed at odds with the hypothesis of one molecule with two independent functional regions. Over the past decade a new notion has emerged that views both Ig regions as interacting and functionally connected. These observations have also shown that V–C interactions may vary between isotype families, suggesting that the basic model of Ig structure–function needs to be reconsidered and revised.

ISOTYPE SWITCHING GENERATES VARIANTS WITH DIFFERENT PROPERTIES

Beginning in the early 1990s and continuing into the first decade of the twenty-first century, at least eight independent groups have reported that isotype switching is associated with altered specificity despite conservation of V region sequence (Kato et al., 1991; Pritsch et al., 1996; McLean et al., 2002; Torres et al., 2005, 2007a; Dam et al., 2008; Torres and Casadevall, 2008; Casadevall and Janda, 2012; Tudor et al., 2012; Xia et al., 2012; Hovenden et al., 2013; Dodev et al., 2015). This effect has been reported for IgM, IgG, IgE and IgA with both protein and nucleic acid Ags, using a variety of techniques such as ELISA, ITC, and SPR (**Table 1**).

An early study from Kato et al., found significant changes in Ag binding when comparing murine isotype Fabs with identical V_L, C_L, and V_H sequences as well as an Fab with a complete C_{H1} domain deletion mutant (Kato et al., 1991). They used ^{13}C-Nuclear Magnetic Resonance (NMR) as a spectroscopic probe of both lateral and longitudinal domain-domain Fab interactions upon Ag binding in a group of murine Fabs derived from IgG_1, IgG_{2a}, and IgG_{2b} isotypes. They were also able to probe chemical shifts directly responsible for Ag binding within the CDR3 loop. They found isotype differences in chemical shifts in the CDR3 region, or paratope as well as other, non-Ag binding residues throughout the Fabs. This work implied that subtle chemical shifts in both the paratope and other, more distant residues occurred within Fab molecules upon Ag binding, and that these shifts could vary depending on isotype (Kato et al., 1991).

Another study, which appeared shortly afterward, used human monomeric Fabs derived from IgA_1 and IgG_1 in Surface Plasmon Resonance (SPR) experiments with a monovalent Ag. Significant differences in Ab–Ag association rate constants between the Fabs studied were found (Pritsch et al., 1996). In this work the authors suggested that in a particular mAb, affinity maturation could be achieved through class switching, suggesting a role for the C_{H1} domain in allosterically influencing the Ag binding site (Pritsch et al., 1996). The same group, in 2000, isolated four human IgG mAbs, three of which had identical V_L and V_H domains. Among this set, the IgA_1 and IgG_1 isotypes manifested significant differences in affinity, though they bound to the same Ag motif. They then created IgG_1 and IgM from the IgA_1 mAb, and found that the IgM bound to its epitope with the same affinity as the parental IgA_1, whereas the IgG_1 had significantly lower affinity as measured by SPR. This result was reproducible using Fabs derived from IgG_1 and IgA_1 (Pritsch et al., 2000). Consequently, they hypothesized that conformational changes in Fab molecules were occurring upon Ag binding, and were most likely being transferred through the elbow angle. Furthermore, Pritsch et al. were able to create a model of the V_H–C_{H1} interface of their IgA_1 and IgG_1 using X-ray crystallographic structures derived from homologous proteins, and found that one of the C_{H1} loops was directly involved in V_H–C_{H1} contacts. Their model also showed that this loop had a different conformation for each isotype (Pritsch et al., 2000). This work supports the hypothesis that the C_{H1} domain has an allosteric role in Ag binding and may also be transmitting structural signals to the rest of the Ig as suggested by Huber et al.'s earlier work.

McLean et al. made chimeric mAb isotypes (murine V region with human IgA_1, IgG 1/2/3/4, and IgM C regions) that showed differences in fine specificity from the parent murine Ab. Furthermore, they saw differences between the various chimeric Abs upon binding monovalent and multivalent Ags, as well as differences in their binding location to a microbial capsule (McLean et al., 2002). Further SPR studies using this family of mAbs compared the intact murine IgG_1 with its chimeric form as well as its deglycosylated form. These studies revealed that glycosylation did not alter Ag binding kinetics and there were no significant differences in Ag binding between the original mAb and its murine–human chimeric form (Torres et al., 2007a). However, it is unclear whether C region glycosylation may play a role in the Ag binding of other families of isotypes, as this has not been thoroughly studied.

A series of studies with the 3E5 family of murine isotypes to *Cryptococcus neoformans* polysaccharide also identified significant changes in isotype specificities and affinities. Using both multi and monovalent Ags, and ELISA, SPR, and Isothermal Titration Calorimetry (ITC) studies, the authors measured

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TABLE 1 | Summary of studies finding mAb isotype differences in Ag binding.

Year	Finding	References
1991	When C_L and C_{H1} residues were substituted in $mIgG_1$, 2a, and 2b Fab isotypes, differences in Ag binding using HSQC NMR were seen.	Kato et al., 1991
1993	Murine IgG_3 isotype was shown to self-aggregate through non-covalent Fc–Fc interactions, thereby causing differences in affinity for and binding to multivalent Ags as compared to its IgG_1 isotype.	Greenspan and Cooper, 1993
1996	hIgA1, $hIgG_1$, $hIgG_2$, and $hIgG_4$ isotypes were found to have different KD constants using SPR.	Pritsch et al., 2000
2002	$mIgG_1$, and mouse-human chimeras (murine V-region with human constant regions 1, 2, 3, 4, M, and A1) were seen to have different Ag binding profiles by ELISA and Immunofluorescence. SPR studies showed no changes in thermodynamics upon deglycosylation of $mIgG_1$, as well as significant differences between mIgG1 and its chimera.	McLean et al., 2002; Torres et al., 2007a
2005–2015	$mIgG_1$, 2a, 2b, and 3 isotypes were shown to have different Ag binding profiles and kinetics using ELISA, Immunofluorescence, ITC and SPR. They were further found to have differences in Trp fluorescence, CD spectroscopy with multivalent Ag, and chemical shifts by NMR when binding to a monovalent Ag. Monovalent peptide Ag cleavage assays revealed different abilities to cleave Ag as well as different rates of cleavage between the isotypes that cleaved.	Torres et al., 2005, 2007b; Janda and Casadevall, 2010; Casadevall and Janda, 2012; Janda et al., 2012, 2015; Eryilmaz et al., 2013
2012	A human IgG_1 and its IgGA2 isotype were found to have differences in HIV-1 gp41 epitopes as well as Ag affinities measured by ELISA.	Tudor et al., 2012
2012–2013	$mIgG_1$, 2a, 2b, and 3 isotypes displayed differences in affinity to the same Ag using SPR methods, as well as isotype differences in Trp fluorescence and CD spectroscopy upon Ag binding.	Xia et al., 2012, 2013
2013	$mIgG_1$, 2a, 2b, and 3 isotypes were found to have different affinities to Ag using SPR.	Hovenden et al., 2013
2013	Human IgG_1 and IgA2 found to have different affinities for HIV-1 Env Ag by SPR.	Tomaras et al., 2013
2015	Human IgG, IgA, IgE isotypes compared by SPR, found to have different Ag affinities.	Dodev et al., 2015

significant differences in binding among the four IgG isotypes (Torres et al., 2005, 2007b; Dam et al., 2008). A monovalent peptide Ag mimetic was used for ELISA binding studies, SPR, and ITC studies. SPR was done with Fabs derived from the 3E5 murine IgG isotype set to isolate the V region and a single C region—the C_{H1}. Among the isotypes, the IgG_1 Fab showed the most favorable binding parameters. This implied that differences in specificity among the isotypes were potentially due to differences in the C_{H1} region alone (Torres et al., 2007b). ITC studies done with the 3E5 family using full IgG molecules and the P1 peptide Ag, confirmed a 2:1 binding stoichiometry of peptide:Ab as well as significantly different association constants between all four isotypes (Dam et al., 2008).

In 2012, Tudor et al. reported increased monovalent Ag specificity and binding affinity when switching an anti-HIV-1 human IgG_1 to a monomeric IgA2. They also found altered epitope specificity and increases in anti-HIV-1 activity assays, indicating significant changes to the Ig paratope (Tudor et al., 2012). A study by Crespillo et al., with the same family of mAbs, compared ITC binding parameters between the Fab and whole IgG of human 2F5. Their results showed significant differences in binding affinities between the different forms of Ab and monomeric peptide Ag epitopes, with highest affinities achieved with whole IgG (Crespillo et al., 2014). This adds to the observation that in this case, in addition to the C_{H1} region, the C_{H2} and/or C_{H3} regions may also be playing a strong role in Ag binding.

More recently, Xia et al. expanded this observation to include anti-nuclear mAbs. They discovered that a family of anti-DNA murine mAb IgG isotypes had significant differences in binding affinities by SPR. They also studied Trp fluorescence and circular dichroism with these isotypes and observed changes upon Ag binding that were isotype dependent (Xia et al., 2013).

For a set of Abs binding to the *Bacillus anthracis* capsule class-switching from the original IgG_3 to IgG_1, IgG_{2a}, and IgG_{2b} isotypes resulted in a loss of protection, affinity and a change in mAb binding to its capsular Ag (Hovenden et al., 2013). Hovenden et al. further determined that switching the C_{H1} region of the protective isotype with highest affinity (IgG_3), for the C_{H1} region from a non-protective isotype with the lowest affinity (IgG_{2b}), showed no loss of affinity or protection. However, swapping the C_{H2} or C_{H3} regions from the IgG_{2b} to the IgG_3, reduced affinity and resulted in a loss of protection, more so with the C_{H2} region (Hovenden et al., 2013). This contrasts with earlier studies that reported similar binding differences between Fab fragments and whole IgG, suggesting an allosteric role for the C_{H1} domain (Yuan et al., 1995, 1998; Torres et al., 2007b). Furthermore, Hovenden et al. used a monovalent peptide to measure fluorescence perturbation as an indication of intrinsic affinity, and found significant differences among the isotypes. Although they could not exclude a contribution from Fc–Fc interactions, there was evidence that other factors must also be contributing to the observed changes in affinity (Hovenden et al., 2013). Using their family of isotypes, they were also able to exclude the hypothesis that as flexibility of the hinge region increases, Ag binding affinity increases. This hypothesis had been suggested by Morelock et al. to explain how isotype could influence human mAb affinity (Morelock et al., 1994; Tomaras et al., 2013). Hovenden et al. further hypothesized that the contributions from the C region in this family of mAb isotypes may be due to (i) glycosylation, although de-glycosylation of

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their IgG_3 did not change its functional affinity, (ii) possible C_{H2} influence on mAb charge, or (iii) C region effects on the chemical and/or electrostatic environment of the paratope. These studies shed light on the different behaviors of different families of mAb isotypes and the need to further investigate and understand how C regions affect paratope properties and how this may differ between different mAb families.

Recently, several studies have identified additional instances where Abs with identical V regions differing in isotype manifest differences in Ag binding. A group studying human anti-HIV-1 Env mAbs identified that an IgA_2 and IgG_1 sharing the same V region had significantly different Env Ag binding affinities using SPR studies. In fact, they found that the IgA_2 bound one Ag strain with 2.9-fold higher magnitude and affinity and another Ag strain with 40-fold greater affinity than the IgG_1 isotype (Tomaras et al., 2013). Another group studying the Phl p7 grass pollen allergen generated a full panel of human IgG and IgA isotypes and found subtle but significant differences in binding rates using SPR. They observed the greatest differences in both on- and off-rate constants to be about three-fold, which canceled out to give an overall affinity range of 250–570 picomoles (pM) (Dodev et al., 2015).

In addition to the various examples of V region-identical Igs exhibiting specificity differences, it is important to note that several studies have reported V region-identical Igs with no changes in specificity. Although the failure to detect changes could be the result of insufficient sensitivity in the assays done, it is possible that the phenomenon of C-mediated changes in specificity and affinity is associated with some V regions and not others. Consequently we compared unique V_H and V_L sequences from 24 Igs with V region-identical isotype switch variants compiled from the literature (**Table 2**). An analysis of the sequence similarities and germline gene hits for these Igs shows that these appear to group phylogenetically, consistently with the notion that certain V region gene families may differ in being permissive or non-permissive of specificity changes following class switching (**Figure 2**). It is interesting to note that all of the human lambda V_L Igs were non-permissive. With the small number of Ig sequences available for this analysis, no firm conclusions can be made and more examples will be needed to confirm a V region germline basis for C domain-mediated specificity changes. It will also be useful to compare the structures of Igs with very similar V regions that differ in specificity to determine whether they are permissive of specificity changes, such as V_L sequences 6, 8, and 11 in **Table 2**. Furthermore, the combination of specific V_H and V_L genes is another potential variable for C domain-mediated specificity changes since it is conceivable that even for permissive V regions that expression of this effect requires combination with certain C regions. In this regard it is noteworthy that different C regions combined with the same V region manifested differences in the magnitude of the changes observed (Janda et al., 2015).

These studies necessitate expanding the role for isotype class switching to a new role where it contributes to the generation of Ig diversity. A recent review by Sela-Culang et al., suggests that changes in C regions or their conformations may lead to structural C domain rearrangement relative to other C and V domains. They suggested that this could re-shape the Ag binding site and as such could be considered as a mechanism for generating Ab diversity (Sela-Culang et al., 2013). This is further supported by the hypothesis that the conformational diversity of Abs is directly linked to Ab multispecificity and supports the role of a single sequence (i.e., V region) adopting multiple structures and functions (Reitan and Hannestad, 1995, 2001; Lange et al., 1996; James and Tawfik, 2003). Since the mechanisms of isotype switching and somatic mutation share some of the same proteins this notion has the elegance of bringing together these two processes within the same molecular pathways. Isotype class switching has further implications for primary and secondary B cell responses, idiotype reactivity and immunogenicity. Also, the observation that Ig class switching can result in reactivity for self Ags despite identical V regions suggests that this phenomenon may be implicated in the appearance of certain pathological autoimmune responses (Torres et al., 2007b). An understanding of how C regions affect Ab paratope is important for the development of therapeutic mAbs (Nosanchuk, 2013). At least two mechanisms have been proposed for this effect: (i) C region-mediated allosteric effects on V region paratope structure before and/or during Ag binding (Huber et al., 1976; Janda et al., 2012) and (ii) subclass-mediated differences in functional affinity leading to the differential recognition of multivalent epitope arrays (Cooper et al., 1993). A plausible mechanism on the molecular level for the first of these effects involves C-mediated structural constraints on V region structure that affect the conformation of the Ig paratope. In contrast, C region glycosylation has thus far not been shown to contribute to this phenomenon (Torres et al., 2007a,b; Hovenden et al., 2013).

C REGION MODIFICATION OF SPECIFICITY THROUGH INCREASED FUNCTIONAL AFFINITY

In 1987, Greenspan et al. reported that murine IgG_3 mAbs specific for the cell wall polysaccharide of *Streptococcus pyogenes* were able to bind cooperatively to whole bacteria treated with a protease to make the relevant epitopes accessible. The Fc regions were found to be required for this enhanced binding. Subsequently, Cooper et al. (1991) demonstrated that IgG_3 bound more strongly to treated (as above) group A streptococcal bacteria than IgG_1 and IgG_{2b} switch variants confirmed to be V domain-identical by cDNA sequencing (Greenspan et al., 1987; Cooper et al., 1991).

Cooper et al. (1993) further demonstrated that in this system the IgG_3 mAb exhibited a different pattern of binding to three strains of group A streptococci displaying the relevant epitope at different densities than the IgG_1 or IgG_{2b} intact Ig molecules or $F(ab')_2$ fragments of IgG_3. While the IgG_1 and IgG_{2b} Abs and the IgG_3 $F(ab')_2$ fragments bound best to the streptococcal strain with the highest epitope density, the IgG_3 Ab bound best to the strain with intermediate epitope density. Additional experiments verified that despite these differences in binding to multivalent Ags, the three Abs (IgG_3, IgG_1, and IgG_{2b}) bound to monovalent Ag or

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TABLE 2 | Unique antibodies with variable-region-identical isotype-switch variants and identifiable amino acid sequences.

seqID	Antibody	Organism	Permissive	Top VL gene	Top VH gene	References
2	HGAC 39.G3, G1, G2b	Murine	Y	IGKV2-109*04	IGHV6-3*01	Greenspan and Cooper, 1993
4	F105 mAb anti-gp120 G1, G3	Human	N	IGKV3-20*01	IGHV4-59*01	Marasco et al., 1992
5	anti-tubulin mAb	Human	Y	IGKV2-28*01	IGHV3-73*02	Pritsch et al., 2000
6	IF6, 1E1, 2E12 mAbs anti-O6 LPS	Murine	N	IGKV1-110*01	IGHV1S137*01	Pollack et al., 1995
8	18B7 anti-GXM	Murine	Y	IGKV1-110*01	IGHV5-6-2*01	Mukherjee, 1992
10	F425 mAb anti-gp140 G2, G1, G3, A	Human	Y	IGKV1D-33*01	IGHV3-64*01	Liu et al., 2008
11	3E5 anti-GXM	Murine	Y	IGKV1-110*01	IGHV5-6-2*01	Mukherjee, 1992
14	F240 mAb anti-HIV G1, G3, G4	Human	N	IGKV4-1*01	IGHV3-11*04	Cavacini et al., 1998
15	F598 mAb anti-PNAG G2, G1	Human	N	IGLV4-69*01	IGHV4-59*01	Kelly-Quintos et al., 2006
16	F628 mAb anti-PNAG G2, G1	Human	N	IGLV4-69*01	IGHV4-59*01	Kelly-Quintos et al., 2006
17	F630 mAb anti-PNAG G2, G1	Human	N	IGLV4-69*01	IGHV1-18*04	Kelly-Quintos et al., 2006
18	2G8, 1E12 mAbs anti-beta glucan G2b, M	Murine	Y	IGKV1-133*01	IGHV1-9*01	Torosantucci et al., 2009
20	12.6 mAb anti-pfMSP1	Murine	Y	IGKV4-70*01	IGHV9-1*02	Porter and Press, 1957
21	12.10 mAb anti-pfMSP1	Murine	Y	IGKV6-17*01	IGHV1S81*02	Porter and Press, 1957
23	2F5 bNAb anti-HIV1 G1, A2	Human	Y	IGKV1-13*02	IGHV2-5*02	Kunert et al., 1998
24	C1 mAb anti-pfMSP1 G1, G2a, G2b, G3	Murine	N	IGKV8-28*01	IGHV1-85*01	Adame-Gallegos et al., 2012
27	IgA1 and IgG1 crystallized Fabs	Human	Y	IGKV2-28*01	IGHV3-73*02	Correa et al., 2013
28	F24F2 mAb anti-γdPGA *B. anthracis* G3, G1, G2a, G2b	Murine	Y	IGKV1-135*01	IGHV10-1*02	Kozel et al., 2007
29	F26G3 mAb anti-γdPGA *B. anthracis* G3, G1, G2a, G2b	Murine	Y	IGKV1-135*01	IGHV10-1*02	Kozel et al., 2007
30	PL9-11 mAb G3, G1, G2a, G2b	Murine	Y	IGKV8-28*02	IGHV5-9*04	Xia et al., 2013
34	FI6 bNAb anti-HA stalk G1, G2a, DA265	Human	N	IGKV4-1*01	IGHV3-30-3*02	Porter, 1957
35	PY102 mAb anti-HA head G1, G2a, DA265	Murine	N	IGKV8-28*01	IGHV5-9*04	Zaghouani et al., 1989
41	PGT121	Human	N	IGLV3-21*02	IGHV4-4*08	Walker et al., 2011
45	20B1 G1, G2a, G2b	Murine	N	IGKV9-124*01	IGHV9-4*02	French et al., 1987

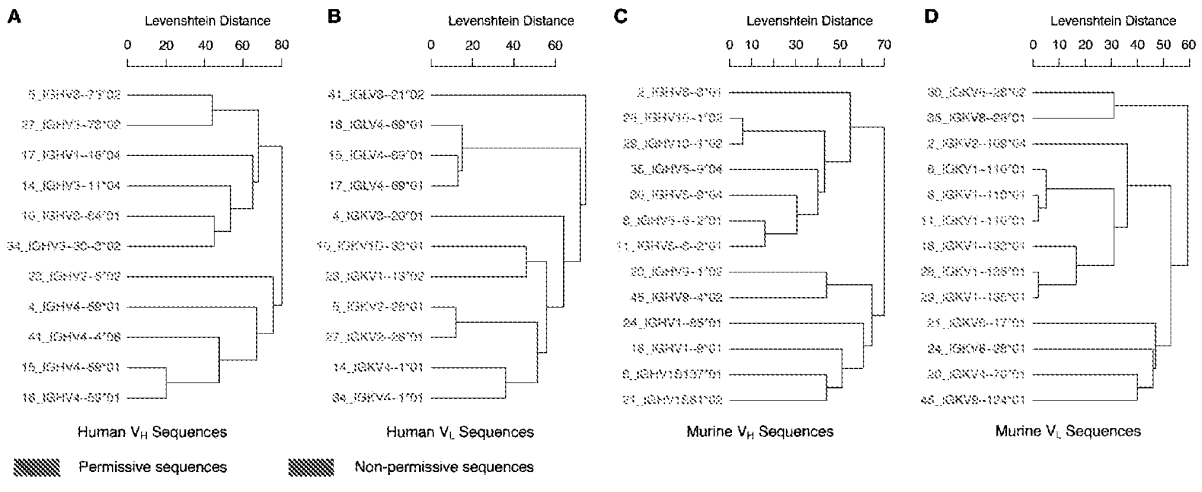


FIGURE 2 | Relationships between permissive and non-permissive V region sequences. Immunoglobulins with differing C regions and identical V regions were identified in the literature (**Table 2**). VH and VL amino acid sequences were found for 24 of these unique antibodies (11 human and 13 murine, **Table 2**). Human sequences were grouped by **(A)** VH and **(B)** VL sequences. Murine sequences were also grouped by **(C)** VH and **(D)** VL sequences. For each group, a dendrogram was constructed through hierarchical average-linkage clustering with pairwise sequence similarity calculated as the Levenshtein distance. Leaf labels in the dendrograms are colored according to whether changes in the constant region for that antibody were permissive (green) or non-permissive (red) of specificity changes. The top V-region gene candidate for each amino acid sequence was determined with the IGBLAST blastp program using default parameters for either the human or mouse database (IGBLAST). Leaf labels in the dendrograms are of the form "seqID_top V-region gene hit," where seqID matches the sequence ID given in **Table 2**.

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multiple rat anti-idiotypic mAbs comparably (Cooper et al., 1993).

Thus, in spite of identical V domain amino acid sequences and ability to bind monovalent hapten, the IgG_3 Ab discriminated among multivalent antigens differently than the IgG_1 or IgG_{2b} Abs (i.e., exhibited different multivalent fine specificities). Additional conclusions drawn by the authors are that epitope density and H chain C region structural differences can contribute to differences in multivalent binding among IgG subclasses. Analysis of this Ab-Ag system with SPR revealed that the stronger binding of the cooperative IgG_3 Ab, in comparison to the non-cooperative IgG_1 and IgG_{2b} Abs, was associated with both greater on rates and slower off rates, consistent with the hypothesis that non-covalent Fc–Fc interactions mediated the cooperativity of the IgG_3 Ab (Cooper et al., 1994).

Also in 1993, Schreiber et al. generated an IgG_3 isotype switch to a murine IgG_1 mAb against a *Pseudomonas aeruginosa* polysaccharide Ag, as well as an IgG_1-derived $F(ab)_2$. These authors found that the intact IgG_3 molecule had stronger binding affinities than the V domain identical IgG_1 Ab to the *P. aeruginosa* Ag, and they hypothesized that this better binding was most likely due to the greater functional affinity (i.e., multivalent affinity or avidity) of the murine IgG_3 Ab (Schreiber et al., 1993).

In roughly the same time period, Izui and colleagues published a number of papers focused on murine IgG_3 cryoglobulins in autoimmune disease models that reported data consistent with the results of Greenspan and his associates. For example, Fulpius et al. studied a pathogenic murine IgG_3 mAb derived from a MRL/MpJ-lpr/lpr mouse and exhibiting cryoglobulin and rheumatoid factor activity for IgG_{2a} along with a V domain-identical switch variant Ab of the IgG_1 subclass. The IgG_1 Ab lacked the cryoglobulin activity and displayed at least a 90% reduction in ability to bind to IgG_{2a} Fc regions (Fulpius et al., 1993).

Another group with similar results on isotype differences in avidity during this time used a set of chimeric mAbs (murine V region, human C region) against both monovalent and bivalent intercellular adhesion molecule 1. They used ELISA competition studies with whole mAb as well as chimeric and murine Fab fragments. Though full-length mAbs showed differences in competition ELISA, their murine and chimeric Fab counterparts had equivalent binding constants, indicating that any differences in whole mAb were due to differences in avidity, and not monovalent affinity (Morelock et al., 1994).

C REGION MODIFICATION OF SPECIFICITY THROUGH ALLOSTERIC CHANGES

Circular dichroism studies done on a family of murine IgGs to *C. neoformans* polysaccharide showed that the C and V regions are structurally coupled and affect each other during Ag binding (Janda and Casadevall, 2010). This was followed by tryptophan fluorescence studies of the same family of murine IgG Abs which showed different changes in electrical properties of Ig Fab Trp molecules some of which are in the paratope, upon Ag binding. NMR studies of the same group of Igs further expanded the notion by showing differences in the chemical environments of their paratopes, as well as IgE and IgA isotypes. Finally, X-ray crystallographic studies and molecular modeling of these Igs identified structural differences that occur mainly in the hinge angles among Fab molecules of the IgG_1 and IgG_3 isotypes (Janda et al., 2012, 2015).

In 2003 Adachi et al. used molecular dynamics simulations to compare the crystal structures of a murine mAb IgG_1 Fab alone, Fab in complex with its hen egg lysozyme (HEL) Ag and the Fv (V regions only) in complex with HEL (Adachi et al., 2003). The Fv–HEL complex was found to have a dissociation constant one order of magnitude lower than that of the Fab–HEL complex (Lavoie et al., 1992). In addition, Adachi et al. identified significant differences in the structures including (i) 18 water molecules in Fv-HEL interface, while the Fab-HEL interface had only one, (ii) the second upper loop in the C_L domain in the Fab light chain (UL2-C_L) showed large conformational fluctuations when compared to the crystal structure of Fab alone. The difference in water molecules in the interface may represent a much tighter interaction for the Fab-HEL complex, which may be essential for HEL binding and could explain the differences in dissociation factors. The authors hypothesized that removal of the C domains in the Fv molecule may result in imperfect complementarity between Ab and Ag, and thus lead to an increase in water molecules between paratope and epitope (Adachi et al., 2003). Furthermore, the UL2-C_L region, which is highly conserved in human and murine light chains, was previously predicted to have unique fluctuations corresponding with Ag binding (Kabat et al., 1975). These new studies indicate that the UL2–C_L fluctuations may be playing an important role in allosteric mediation of paratope–epitope interactions (Adachi et al., 2003).

In 2013, Xia et al. found that anti-DNA mAb V-domain identical switch variants from a murine SLE autoantibody model have different changes in secondary structure upon Ag binding, confirming coupling of the C and V regions. In addition, they found significant differences in histone and kidney Ag binding profiles using SPR, and different changes in Trp fluorescence upon Ag binding. Moreover, these differences were associated with significant differences in renal pathogenicity and survival studies that included *in-vivo* Ab administration (Xia et al., 2013).

X-ray crystallographic studies of V region identical human Fab from IgA_1 and IgG_1 by Correa et al. reveal greater rigidity in the C_{H1}–C_L and C_{H1}–V_H interfaces in the IgA_1 structure, suggesting that these could exert allosteric effects on the paratope. They identified a large hydrophobic core of residues in the IgA1 V_H–C_{H1} interface, as well as a disulfide bridge connecting heavy and light chains which was absent in the IgG_1 model. Furthermore, they identified a difference of about 5° in the angle between the V_H–C_{H1} domains of the two Fabs which modifies the V_H–V_L arrangement. This subtle change may result in allosteric effects that lead to critical rearrangements of the paratope. These constraints ultimately led to an increased rigidity of the IgA1 molecule and different conformational entropy that is hypothesized to correlate to Ag binding affinity modulation (Correa et al., 2013).

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A comparison of human and murine mAb IgG isotypes using the full ensemble optimization method and SAXS data of these isotypes by two separate groups revealed important conformational differences (Eryilmaz et al., 2013; Tian et al., 2015). Eryilmaz et al. proposed that differences in global structures of isotypes are due to cross-domain relationships between various V and C region combinations. They hypothesized that these relationships can dramatically change the overall shape of an Ig (Eryilmaz et al., 2013). This was supported by studies by Tian et al. which showed that differences seen in Ag binding may be related to intermolecular Fab–Fab and Fab–Fc interactions. This was based on the significant differences in hinge angles and the type of overall conformation that was adopted by the IgG molecules they used (Tian et al., 2015).

Recent studies comparing 141 crystal structures of Abs with and without Ag provided additional insight into C region involvement in Ag binding. These studies show that Ag binding is associated with changes in (1) H and L chain relative orientations in both C and V domains, (2) elbow angles between V and C regions, especially when binding large Ags, and (3) C_{H1} loops implicated in interactions between H and L chains which show the most consistent and substantial changes upon Ag binding. Although the group only compared pairs of structures solved with the same space group, no two pairs had similar unit cell sizes. However, these results were consistent among many pairs of Fabs and their Fab-Ag structures, and provide some insight into how C-region mediated allosteric effects could potentially transmit to the V region (Sela-Culang et al., 2013).

ANTIGEN-INDUCED CHANGES IN FC REGION

It is also worth mentioning that there are a few studies that have hypothesized allosteric changes in Fc regions upon Ag binding, thus promoting Ab effector functions. This strengthens the argument for Ig inter-molecular signaling through specific structural interactions. For example, in 1970's Brown and Koshland identified changes in J-chain exposure in C_{H4} Fc regions of IgM molecules that were directly induced by monovalent Ag binding to the Fab region. Their studies excluded cross-linked Ags (Brown and Koshland, 1975, 1977). In addition, studies by Schlessinger et al. using Trp fluorescence and monovalent Ags with anti-RNase Abs demonstrated significantly different C-region changes in both Fab and whole Ab upon Ag binding. This suggests an interaction between Ag binding sites and distant Fc regions. When they reduced the inter-chain disulfide bonds of the Fabs and whole Abs, they no longer saw these changes in whole Ab molecules. This work indicates that disulfide bonding especially at the Ab hinge region is required for the transmission of allosteric signaling from the Fab region to the Fc in these Ab molecules (Schlessinger et al., 1975). Another study, which assessed IgG Fc binding to staphylococcal protein A and streptococcal protein G proteins (which bind to Ig C domains), identified inhibition of binding in the presence of Ag, presumably through changes to the Fc region. These observations were also made in the presence of a reducing environment (Oda et al., 2003). These studies raise the question of Ig allosteric effects that occur after Ag binding and travel down the molecule to induce changes in the Ig Fc region.

CONCLUDING REMARKS

Since the initial findings that Ig C regions can change the Ag binding parameters of their V regions, more recent studies have begun to attempt to elucidate the allosteric mechanisms through which these effects may be occurring. Many studies have now shown that inter-molecular interactions between heavy and light chains, V and C regions, hinges and elbow relationships, all can play a vital role in the overall molecular structure of the Ig paratope in relationship to its affinity and specificity for Ag. In particular, the C_{H1} region seems to play a large role in determining Ag binding parameters, most likely through its intermolecular interactions with both its neighboring C_L domain as well as by transmitting structural information to the Ig hinge region and back to the V_H domain.

AUTHOR CONTRIBUTIONS

AJ wrote the manuscript, **Table 1**, and edited the document to prepare for submission. AB put together **Figures 1**, **2**, and **Table 2** and also edited the paper. NG contributed with writing and editing the review. AC is the corresponding author, he wrote and edited the review.

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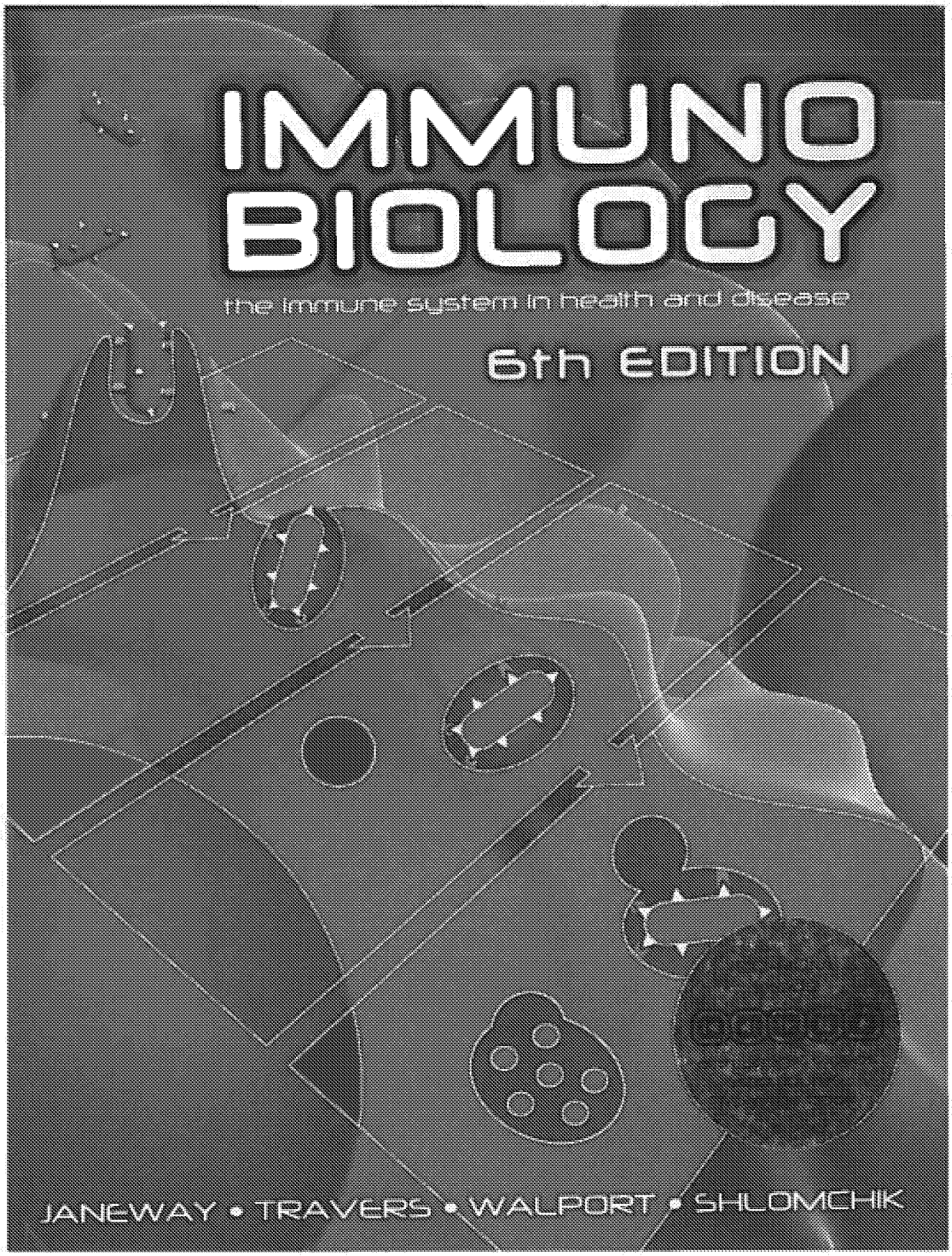
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Antigen Recognition by B-cell and T-cell Receptors

3

We have learned in Chapter 2 that the body is defended by innate immune responses, but these only work to control pathogens that have certain molecular patterns or that induce interferons and other secreted yet nonspecific defenses. Most crucially, they do not allow memory to form because they operate through receptors that are coded in the genome. Thus, innate immunity is good for preventing pathogens from growing freely in the body, but it does not lead to the most important feature of adaptive immunity, which is long-lasting memory of a specific pathogen.

To fight the wide range of pathogens an individual will encounter, the lymphocytes of the adaptive immune system have evolved to recognize a great variety of different antigens from bacteria, viruses, and other disease-causing organisms. The antigen-recognition molecules of B cells are the immunoglobulins, or Ig. These proteins are produced by B cells in a vast range of antigen specificities, each B cell producing immunoglobulin of a single specificity (see Sections 1-8 to 1-10). Membrane-bound immunoglobulin on the B-cell surface serves as the cell's receptor for antigen, and is known as the B-cell receptor (BCR). Immunoglobulin of the same antigen specificity is secreted as antibody by terminally differentiated B cells—the plasma cells. The secretion of antibodies, which bind pathogens or their toxic products in the extracellular spaces of the body, is the main effector function of B cells in adaptive immunity.

Antibodies were the first molecules involved in specific immune recognition to be characterized, and are still the best understood. The antibody molecule has two separate functions: one is to bind specifically to molecules from the pathogen that elicited the immune response; the other is to recruit other cells and molecules to destroy the pathogen once the antibody is bound to it. For example, binding by antibody neutralizes viruses and marks pathogens for destruction by phagocytes and complement, as described in Section 1-15. These functions are structurally separated in the antibody molecule, one part of which specifically recognizes and binds to the pathogen or antigen whereas the other engages different effector mechanisms. The antigen-binding region varies extensively between antibody molecules and is thus known as the variable region or V region. The variability of antibody molecules allows each antibody to bind a different specific antigen, and the total repertoire of antibodies made by a single individual is large enough to ensure that virtually any structure can be recognized. The region of the antibody molecule that engages the effector functions of the immune system does not vary in the same way and is thus known as the constant region or C region. It comes in five main forms, which are each specialized for activating different effector mechanisms. The membrane-bound B-cell receptor does not have these effector functions, because the C region remains inserted in the membrane of the

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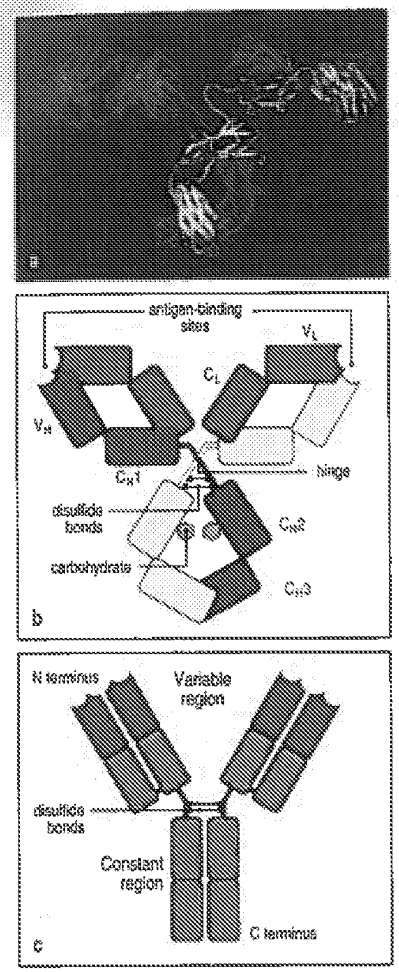


Fig. 3.1 Structure of an antibody molecule. Panel a illustrates a ribbon diagram based on the X-ray crystallographic structure of an IgG antibody, showing the course of the backbones of the polypeptide chains. Three globular regions form a Y shape. The two antigen-binding sites are at the tips of the arms, which are tethered to the trunk of the Y by a flexible hinge region. A schematic representation of the structure in a is given in panel b, illustrating the four-chain composition and the separate domains comprising each chain. Panel c shows a simplified schematic representation of an antibody molecule that will be used throughout this book. C terminus, carboxy terminus; N terminus, amino terminus. Panel a courtesy of A. McPherson and L. Harris.

B cell. Its function is as a receptor that recognizes and binds antigen by the V regions exposed on the surface of the cell, thus transmitting a signal that causes B-cell activation, leading to clonal expansion and specific antibody production.

The antigen-recognition molecules of T cells are made solely as membrane-bound proteins and function only to signal T cells for activation. These T-cell receptors (TCRs) are related to immunoglobulins both in their protein structure—having both V and C regions—and in the genetic mechanism that produces their great variability (see Section 1-11 and Chapter 4). However, the T-cell receptor differs from the B-cell receptor in an important way: it does not recognize and bind antigen directly, but instead recognizes short peptide fragments of pathogens' protein antigens, which are bound to MHC molecules on the surfaces of other cells.

The MHC molecules are glycoproteins encoded in the large cluster of genes known as the major histocompatibility complex (MHC) (see Sections 1-17 and 1-18). Their most striking structural feature is a cleft running across their outermost surface, in which a variety of peptides can be bound. As we shall discuss further in Chapter 5, MHC molecules show great genetic variation in the population. Most people are heterozygous for the MHC molecules: they express two allelic forms of each MHC locus, which increases the range of pathogen-derived peptides that can be bound. T-cell receptors recognize features of both the peptide antigen and the MHC molecule to which it is bound. This introduces an extra dimension to antigen recognition by T cells, known as MHC restriction, because any given T-cell receptor is specific not simply for a foreign peptide antigen but for a unique combination of a peptide and a particular MHC molecule. The ability of T-cell receptors to recognize MHC molecules, and their selection during T-cell development for the ability to recognize the particular MHC molecules expressed by an individual, are topics we shall return to in Chapters 5 and 7.

In this chapter we focus on the structure and antigen-binding properties of immunoglobulins and T-cell receptors. Although B cells and T cells recognize foreign molecules in two distinct fashions, the receptor molecules they use for this task are very similar in structure. We will see how this basic structure can accommodate great variability in antigen specificity, and how it enables immunoglobulins and T-cell receptors to perform their functions as the antigen-recognition molecules of the adaptive immune response.

The structure of a typical antibody molecule.

Antibodies are the secreted form of the B-cell receptor. An antibody is identical to the B-cell receptor of the cell that secretes it except for a small portion of the carboxy terminus of the heavy-chain C region. In the case of the B-cell receptor the carboxy terminus is a hydrophobic membrane-anchoring sequence, and in the case of antibody it is a hydrophilic sequence that allows secretion. As they are soluble, and secreted in large quantities, antibodies are easily obtainable and easily studied. For this reason, most of what we know about the B-cell receptor comes from the study of antibodies.

Antibody molecules are roughly Y-shaped molecules consisting of three equal-sized portions, connected by a flexible tether. Three schematic representations of antibody structure, which has been determined by X-ray crystallography, are shown in Fig. 3.1. The aim of this part of the chapter is to

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explain how this structure is formed and how it allows antibody molecules to perform their dual tasks—binding on the one hand to a wide variety of antigens, and on the other hand to a limited number of effector molecules and cells. As we will see, each of these tasks is performed by separable parts of the molecule. The two arms of the Y end in regions that vary between different antibody molecules—the V regions. These are involved in antigen binding, whereas the stem of the Y, or the C region, is far less variable and is the part that interacts with effector cells and molecules.

All antibodies are constructed in the same way from paired heavy and light polypeptide chains, and the generic term immunoglobulin is used for all such proteins. Within this general category, however, five different classes of immunoglobulins—IgM, IgD, IgG, IgA, and IgE—can be distinguished by their C regions, which will be described more fully in Chapter 4. More subtle differences confined to the V region account for the specificity of antigen binding. We will use the IgG antibody molecule as an example to describe the general structural features of immunoglobulins.

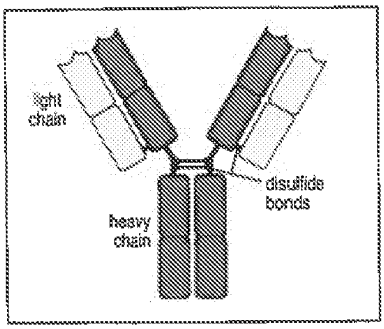


Fig. 3.2 Immunoglobulin molecules are composed of two types of protein chain: heavy chains and light chains. Each immunoglobulin molecule is made up of two heavy chains (green) and two light chains (yellow) joined by disulfide bonds so that each heavy chain is linked to a light chain and the two heavy chains are linked together.

3-1 IgG antibodies consist of four polypeptide chains.

IgG antibodies are large molecules with a molecular weight of approximately 150 kDa and are composed of two different kinds of polypeptide chain. One, of approximately 50 kDa, is termed the **heavy** or **H chain**, and the other, of 25 kDa, is termed the **light** or **L chain** (Fig. 3.2). Each IgG molecule consists of two heavy chains and two light chains. The two heavy chains are linked to each other by disulfide bonds and each heavy chain is linked to a light chain by a disulfide bond. In any given immunoglobulin molecule, the two heavy chains and the two light chains are identical, giving an antibody molecule two identical antigen-binding sites (see Fig. 3.1), and thus the ability to bind simultaneously to two identical structures.

Two types of light chain, termed lambda (λ) and kappa (κ), are found in antibodies. A given immunoglobulin has either κ chains or λ chains, never one of each. No functional difference has been found between antibodies having λ or κ light chains, and either type of light chain can be found in antibodies of any of the five major classes. The ratio of the two types of light chain varies from species to species. In mice, the average κ to λ ratio is 20:1, whereas in humans it is 2:1 and in cattle it is 1:20. The reason for this variation is unknown. Distortions of this ratio can sometimes be used to detect the abnormal proliferation of a clone of B cells. These would all express the identical light chain, and thus an excess of λ light chains in a person might indicate the presence of a B-cell tumor producing λ chains.

By contrast, the class, and thus the effector function, of an antibody is defined by the structure of its heavy chain. There are five main heavy-chain classes or isotypes, some of which have several subtypes, and these determine the functional activity of an antibody molecule. The five major classes of immunoglobulin are **immunoglobulin M (IgM)**, **immunoglobulin D (IgD)**, **immunoglobulin G (IgG)**, **immunoglobulin A (IgA)**, and **immunoglobulin E (IgE)**. Their heavy chains are denoted by the corresponding lower-case Greek letter (μ, δ, γ, α, and ε, respectively). IgG is by far the most abundant immunoglobulin and has several subclasses (IgG1, 2, 3, and 4 in humans). Their distinctive functional properties are conferred by the carboxy-terminal part of the heavy chain, where it is not associated with the light chain. We will describe the structure and functions of the different heavy-chain isotypes in Chapter 4. The general structural features of all the isotypes are similar, and we will consider IgG, the most abundant isotype in plasma, as a typical antibody molecule.

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3-2 Immunoglobulin heavy and light chains are composed of constant and variable regions.

The amino acid sequences of many immunoglobulin heavy and light chains have been determined and reveal two important features of antibody molecules. First, each chain consists of a series of similar, although not identical, sequences, each about 110 amino acids long. Each of these repeats corresponds to a discrete, compactly folded region of protein structure known as a protein domain. The light chain is made up of two such immunoglobulin domains, whereas the heavy chain of the IgG antibody contains four (see Fig. 3.1a). This suggests that the immunoglobulin chains have evolved by repeated duplication of an ancestral gene corresponding to a single domain.

The second important feature revealed by comparisons of amino acid sequences is that the amino-terminal sequences of both the heavy and light chains vary greatly between different antibodies. The variability in sequence is limited to approximately the first 110 amino acids, corresponding to the first domain, whereas the remaining domains are constant between immunoglobulin chains of the same isotype. The amino-terminal variable domains (V domains) of the heavy and light chains (V_H and V_L, respectively) together make up the V region of the antibody and confer on it the ability to bind specific antigen, whereas the constant domains (C domains) of the heavy and light chains (C_H and C_L, respectively) make up the C region (see Fig. 3.1b, c). The multiple heavy-chain C domains are numbered from the amino-terminal end to the carboxy terminus, for example C_H1, C_H2, and so on.

3-3 The antibody molecule can readily be cleaved into functionally distinct fragments.

The protein domains described above associate to form larger globular domains. Thus, when fully folded and assembled, an antibody molecule comprises three equal-sized globular portions joined by a flexible stretch of polypeptide chain known as the hinge region (see Fig. 3.1b). Each arm of this Y-shaped structure is formed by the association of a light chain with the amino-terminal half of a heavy chain, whereas the trunk of the Y is formed by the pairing of the carboxy-terminal halves of the two heavy chains. The association of the heavy and light chains is such that the V_H and V_L domains are paired, as are the C_H1 and C_L domains. The C_H3 domains pair with each other but the C_H2 domains do not interact; carbohydrate side chains attached to the C_H2 domains lie between the two heavy chains. The two antigen-binding sites are formed by the paired V_H and V_L domains at the ends of the two arms of the Y (see Fig. 3.1b).

Proteolytic enzymes (proteases) that cleave polypeptide sequences have been used to dissect the structure of antibody molecules and to determine which parts of the molecule are responsible for its various functions. Limited digestion with the protease papain cleaves antibody molecules into three fragments (Fig. 3.3). Two fragments are identical and contain the antigen-binding activity. These are termed the Fab fragments, for Fragment antigen binding. The Fab fragments correspond to the two identical arms of the antibody molecule, each of which consists of a complete light chain paired with the V_H and C_H1 domains of a heavy chain. The other fragment contains no antigen-binding activity but was originally observed to crystallize readily, and for this reason was named the Fc fragment, for Fragment crystallizable. This fragment corresponds to the paired C_H2 and C_H3 domains and is the part of the antibody molecule that interacts with effector molecules and cells. The functional differences between heavy-chain isotypes lie mainly in the Fc fragment.

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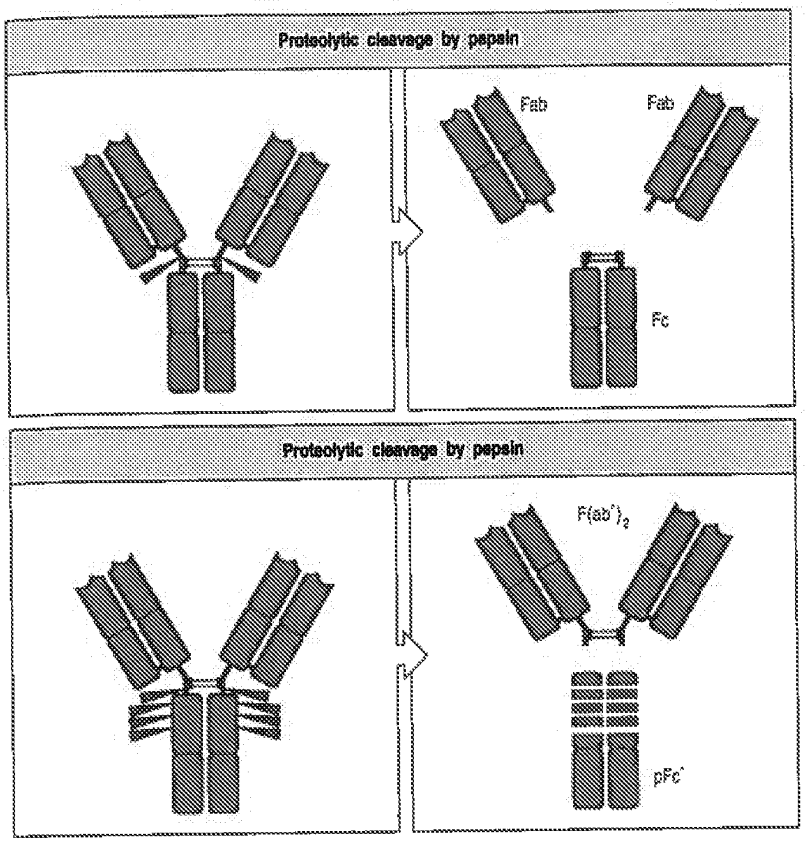


Fig. 3.3 The Y-shaped immunoglobulin molecule can be dissected by partial digestion with proteases. Upper panels: papain cleaves the immunoglobulin molecule into three pieces, two Fab fragments and one Fc fragment. The Fab fragment contains the V regions and binds antigen. The Fc fragment is crystallizable and contains C regions. Lower panels: pepsin cleaves immunoglobulin to yield one $F(abf)_2$ fragment and many small pieces of the Fc fragment, the largest of which is called the pFc0fragment. $F(abf)_2$ is written with a prime because it contains a few more amino acids than Fab, including the cysteines that form the disulfide bonds.

The protein fragments obtained after proteolysis are determined by where the protease cuts the antibody molecule in relation to the disulfide bonds that link the two heavy chains. These lie in the hinge region between the C_H1 and C_H2 domains and, as illustrated in Fig. 3.3, papain cleaves the antibody molecule on the amino-terminal side of the disulfide bonds. This releases the two arms of the antibody as separate Fab fragments, whereas in the Fc fragment the carboxy-terminal halves of the heavy chains remain linked.

Another protease, pepsin, cuts in the same general region of the antibody molecule as papain but on the carboxy-terminal side of the disulfide bonds (see Fig. 3.3). This produces a fragment, the $F(ab')_2$ fragment, in which the two antigen-binding arms of the antibody molecule remain linked. In this case the remaining part of the heavy chain is cut into several small fragments. The $F(ab')_2$ fragment has exactly the same antigen-binding characteristics as the original antibody but is unable to interact with any effector molecule. It is thus of potential value in therapeutic applications of antibodies as well as in research into the functional role of the Fc portion.

Genetic engineering techniques also now permit the construction of many different antibody-related molecules. One important type is a truncated Fab comprising only the V domain of a heavy chain linked by a stretch of synthetic peptide to a V domain of a light chain. This is called single-chain Fv, named from Fragment variable. Fv molecules may become valuable therapeutic agents because of their small size, which allows them to penetrate tissues readily. They can be coupled to protein toxins to yield immunotoxins with potential application, for example, in tumor therapy in the case of a Fv specific for a tumor antigen (see Chapter 14).

3-4 The immunoglobulin molecule is flexible, especially at the hinge region.

The hinge region that links the Fc and Fab portions of the antibody molecule is in reality a flexible tether, allowing independent movement of the two Fab arms, rather than a rigid hinge. This has been demonstrated by electron microscopy of antibodies bound to haptens. These are small molecules of various sorts, typically about the size of a tyrosine side chain. They can be recognized by antibody but are able to stimulate the production of anti-hapten antibodies only when linked to a larger protein carrier (see Appendix I, Section A-1). An antigen made of two identical hapten molecules joined by a short flexible region can link two or more anti-hapten antibodies, forming dimers, trimers, tetramers, and so on, which can be seen by electron microscopy (Fig. 3.4). The shapes formed by these complexes demonstrate that antibody molecules are flexible at the hinge region. Some flexibility is also found at the junction between the V and C domains, allowing bending and rotation of the V domain relative to the C domain. For example, in the antibody molecule shown in Fig. 3.1a, not only are the two hinge regions clearly bent differently, but the angle between the V and C domains in each of the two Fab arms is also different. This range of motion has led to the junction between the V and C domains being referred to as a 'molecular ball-and-socket joint.' Flexibility at both the hinge and the V–C junction enables the binding of both arms of an antibody molecule to sites that are various distances apart, for example, sites on bacterial cell-wall polysaccharides. Flexibility at the hinge also enables the antibodies to interact with the antibody-binding proteins that mediate immune effector mechanisms.

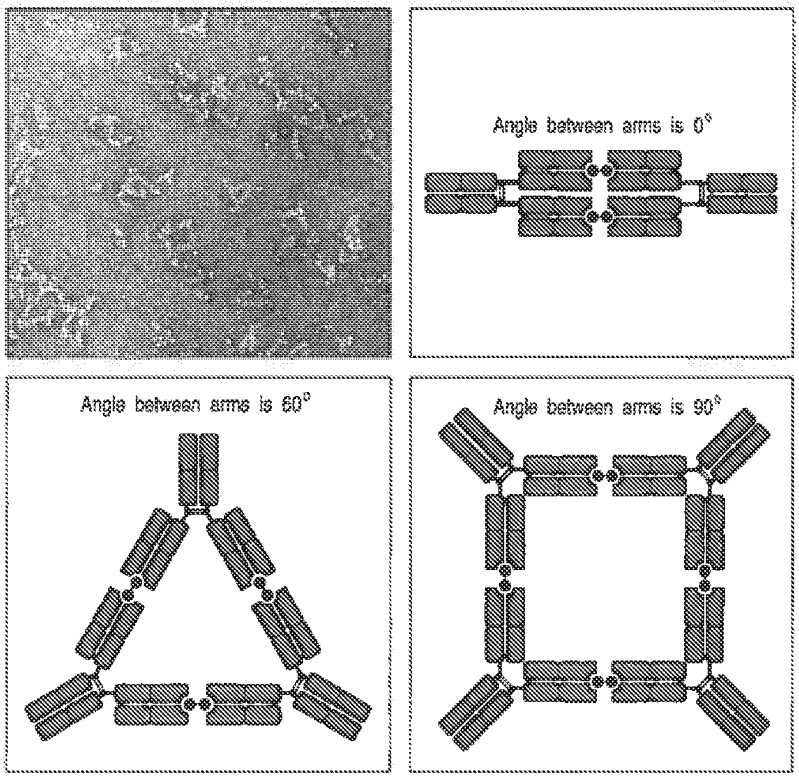


Fig. 3.4 Antibody arms are joined by a flexible hinge. An antigen consisting of two hapten molecules (red balls in diagrams) that can cross-link two antigen-binding sites is used to create antigen:antibody complexes, which can be seen in the electron micrograph. Linear, triangular, and square forms are seen, with short projections or spikes. Limited pepsin digestion removes these spikes (not shown in the figure), which therefore correspond to the Fc portion of the antibody; the $F(ab')_2$ pieces remain cross-linked by antigen. The interpretation of the complexes is shown in the diagrams. The angle between the arms of the antibody molecules varies, from 0° in the antibody dimers, through 60° in the triangular forms, to 90° in the square forms, showing that the connections between the arms are flexible. Photograph (× 300,000) courtesy of N.M. Green.

3-5 The domains of an immunoglobulin molecule have similar structures.

As we saw in Section 3-2, immunoglobulin heavy and light chains are composed of a series of discrete protein domains, all of which have a similar folded structure. Within this basic three-dimensional structure there are distinct differences between V and C domains. The structural similarities and differences can be seen in the diagram of a light chain in Fig. 3.5. Each domain is constructed from two β sheets, which are elements of protein structure made up of strands of the polypeptide chain (β strands) packed together; the sheets are linked by a disulfide bridge and together form a roughly barrel-shaped structure, known as a β barrel. The distinctive folded structure of the immunoglobulin protein domain is known as the **immunoglobulin fold.**

Both the essential similarity of V and C domains and the critical difference between them are most clearly seen in the bottom panels of Fig. 3.5, where the cylindrical domains are opened out to reveal how the polypeptide chain folds to create each of the β sheets and how it forms flexible loops as it changes direction. The main difference between the V and C domains is that the V domain is larger, with an extra loop. We will see in Section 3-6 that the flexible loops of the V domains form the antigen-binding site of the immunoglobulin molecule.

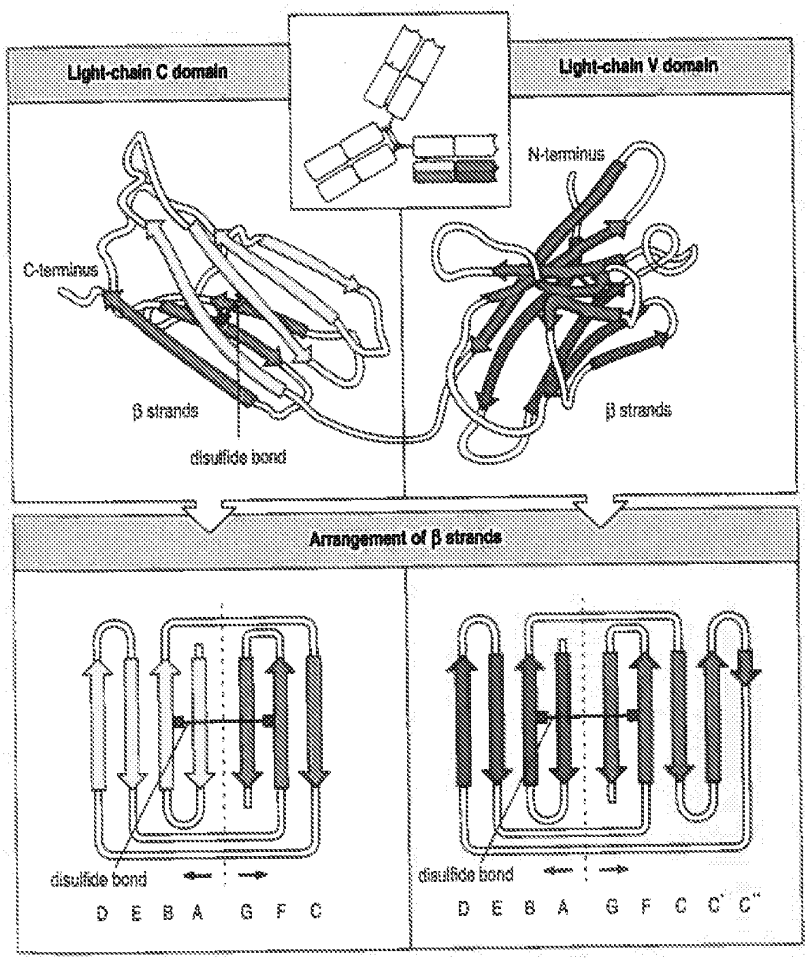


Fig. 3.5 The structure of immunoglobulin constant and variable domains. The upper panels show schematically the folding pattern of the constant (C) and variable (V) domains of an immunoglobulin light chain. Each domain is a barrel-shaped structure in which strands of polypeptide chain (β strands) running in opposite directions (antiparallel) pack together to form two β sheets (shown in yellow and green in the diagram of the C domain), which are held together by a disulfide bond. The way the polypeptide chain folds to give the final structure can be seen more clearly when the sheets are opened out, as shown in the lower panels. The β strands are lettered sequentially with respect to the order of their occurrence in the amino acid sequence of the domains; the order in each β sheet is characteristic of immunoglobulin domains. The β strands C′ and C″ that are found in the V domains but not in the C domains are indicated by a blue shaded background. The characteristic four-strand plus three-strand (C-region type domain) or four-strand plus five-strand (V-region type domain) arrangements are typical immunoglobulin superfamily domain building blocks, found in a whole range of other proteins as well as antibodies and T-cell receptors.

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Many of the amino acids that are common to C and V domains of immunoglobulin chains lie in the core of the immunoglobulin fold and are essential for its stability. For that reason, other proteins having sequences similar to those of immunoglobulins are believed to form domains of similar structure, and in many cases this has been demonstrated by crystallography. These immunoglobulin-like domains are present in many other proteins of the immune system, and in proteins involved in cell–cell recognition in the nervous system and other tissues. Together with the immunoglobulins and the T-cell receptors, they make up the extensive immunoglobulin superfamily.

Summary.

The IgG antibody molecule is made up of four polypeptide chains, comprising two identical light chains and two identical heavy chains, and can be thought of as forming a flexible Y-shaped structure. Each of the four chains has a variable (V) region at its amino terminus, which contributes to the antigen-binding site, and a constant (C) region, which determines the isotype. The isotype of the heavy chain determines the functional properties of the antibody. The light chains are bound to the heavy chains by many noncovalent interactions and by disulfide bonds, and the V regions of the heavy and light chains pair in each arm of the Y to generate two identical antigen-binding sites, which lie at the tips of the arms of the Y. The possession of two antigen-binding sites allows antibody molecules to cross-link antigens and to bind them much more stably. The trunk of the Y, called the Fc fragment, is composed of the carboxy-terminal domains of the heavy chains. Joining the arms of the Y to the trunk are the flexible hinge regions. The Fc fragment and hinge regions differ in antibodies of different isotypes, thus determining their functional properties. However, the overall organization of the domains is similar in all isotypes.

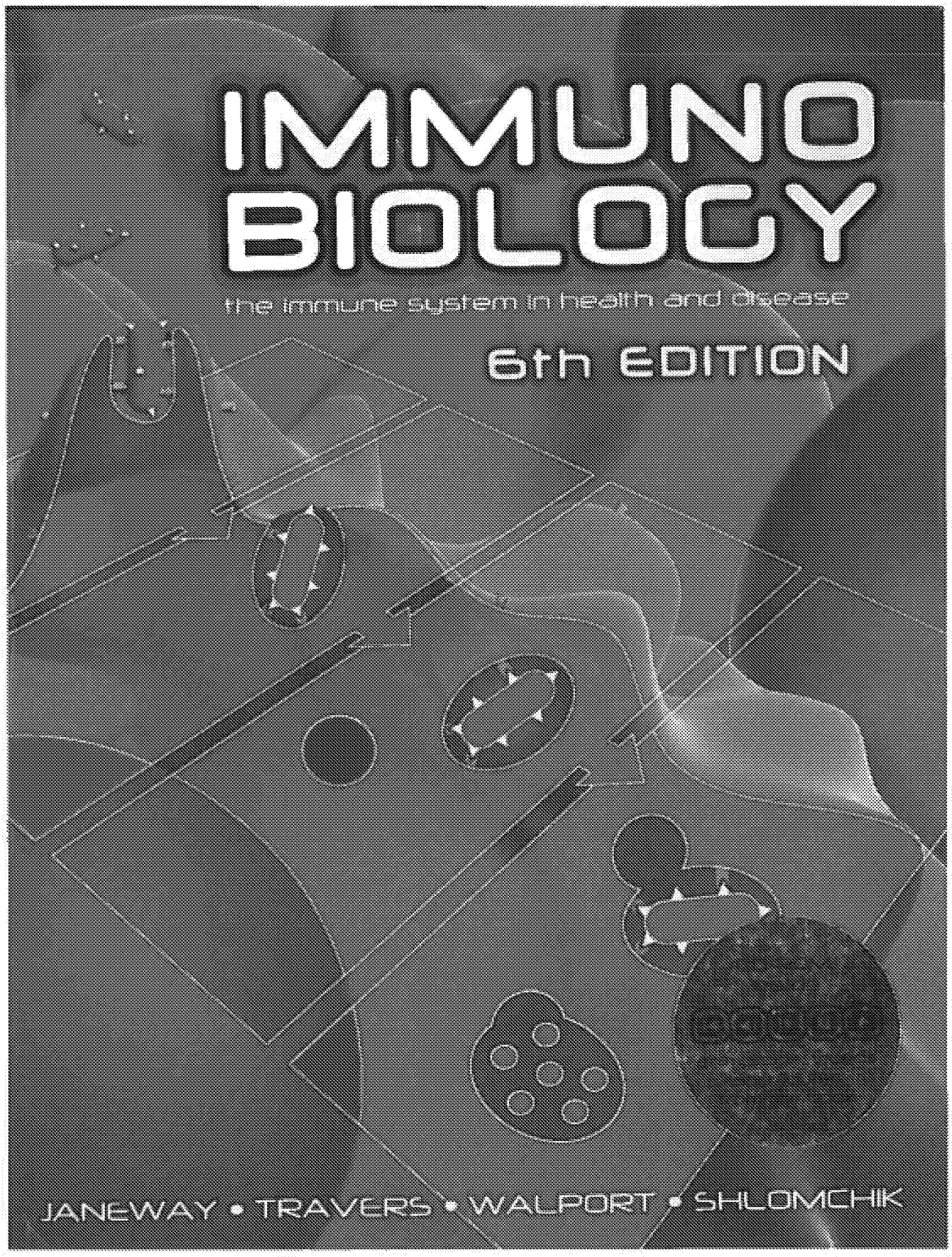
The interaction of the antibody molecule with specific antigen.

We have described the structure of the antibody molecule and how the V regions of the heavy and light chains fold and pair to form the antigen-binding site. In this part of the chapter we look at the antigen-binding site in more detail. We discuss the different ways in which antigens can bind to antibody and address the question of how variation in the sequences of the antibody V domains determines the specificity for antigen.

3-6 Localized regions of hypervariable sequence form the antigen-binding site.

The V regions of any given antibody molecule differ from those of every other. Sequence variability is not, however, distributed evenly throughout the V region but is concentrated in certain segments. The distribution of variable amino acids can be seen clearly in what is termed a variability plot (Fig. 3.6), in which the amino acid sequences of many different antibody V regions are compared. Three particularly variable segments can be identified in both the V_H and V_L domains. They are designated hypervariable regions and are denoted HV1, HV2, and HV3. In the heavy chains these are

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IMMUNOBIOLOGY

the immune system in health and disease

6th EDITION

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the immune system in health and disease

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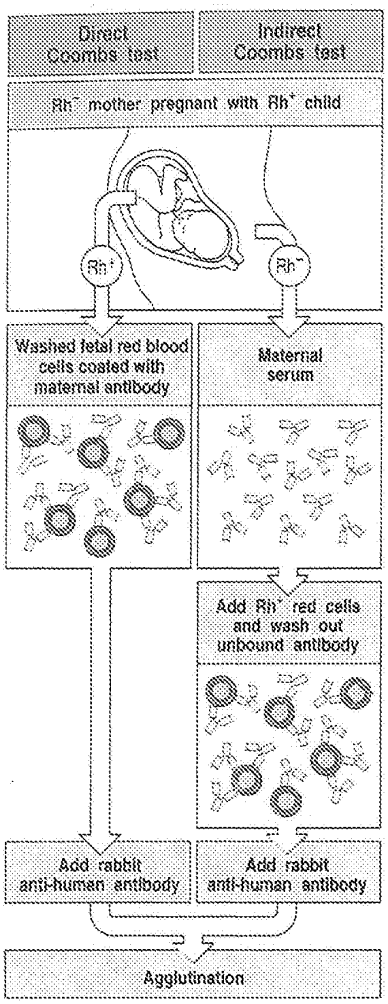


Fig. A.13 The Coombs direct and indirect anti-globulin tests for antibody to red blood cell antigens. A Rh⁻ mother of a Rh⁺ fetus can become immunized to fetal red blood cells that enter the maternal circulation at the time of delivery. In a subsequent pregnancy with a Rh⁺ fetus, IgG anti-Rh antibodies can cross the placenta and damage the fetal red blood cells. In contrast to anti-Rh antibodies, maternal anti-ABO antibodies are of the IgM isotype and cannot cross the placenta, and so do not cause harm. Anti-Rh antibodies do not agglutinate red blood cells but their presence on the fetal red blood cell surface can be shown by washing away unbound immunoglobulin and then adding antibody to human immunoglobulin, which agglutinates the antibody-coated cells. Anti-Rh antibodies can be detected in the mother's serum in an indirect Coombs test; the serum is incubated with Rh⁺ red blood cells, and once the antibody binds, the red blood cells are treated as in the direct Coombs test.

Since the Rh antigens are widely spaced on the red blood cell surface, the IgG anti-Rh antibodies cannot fix complement and cause lysis of red blood cells *in vitro*. Furthermore, for reasons that are not fully understood, antibodies to Rh blood group antigens do not agglutinate red blood cells as do antibodies to the ABO blood group antigens. Thus, detecting these antibodies was difficult until anti-human immunoglobulin antibodies were developed. With these, maternal IgG antibodies bound to the fetal red blood cells can be detected after washing the cells to remove unbound immunoglobulin that is present in the fetal serum. Adding anti-human immunoglobulin antibodies to the washed fetal red blood cells agglutinates any cells to which maternal antibodies are bound. This is the **direct Coombs test** (Fig. A.13), so called because it directly detects antibody bound to the surface of the fetal red blood cells. An **indirect Coombs test** is used to detect nonagglutinating anti-Rh antibody in maternal serum; the serum is first incubated with Rh-positive red blood cells, which bind the anti-Rh antibody, after which the antibody-coated cells are washed to remove unbound immunoglobulin and are then agglutinated with anti-immunoglobulin antibody (see Fig. A.13). The indirect Coombs test allows Rh incompatibilities that might lead to hemolytic disease of the newborn to be detected, and this knowledge allows the disease to be prevented (see Section 10-25). The Coombs test is also commonly used to detect antibodies to drugs that bind to red blood cells and cause hemolytic anemia.

A-12 Monoclonal antibodies.

The antibodies generated in a natural immune response or after immunization in the laboratory are a mixture of molecules of different specificities and affinities. Some of this heterogeneity results from the production of antibodies that bind to different epitopes on the immunizing antigen, but even antibodies directed at a single antigenic determinant such as a hapten can be markedly heterogeneous, as shown by **isoelectric focusing**. In this technique, proteins are separated on the basis of their isoelectric point, the pH at which their net charge is zero. By electrophoresing proteins in a pH gradient for long enough, each molecule migrates along the pH gradient until it reaches the pH at which it is neutral and is thus concentrated (focused) at that point. When antiserum containing anti-hapten antibodies is treated in this way and then transferred to a solid support such as nitrocellulose paper, the anti-hapten antibodies can be detected by their ability to bind labeled hapten. The binding of antibodies of various isoelectric points to the hapten shows that even antibodies that bind the same antigenic determinant can be heterogeneous.

Antisera are valuable for many biological purposes but they have certain inherent disadvantages that relate to the heterogeneity of the antibodies they contain. First, each antiserum is different from all other antisera, even if

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raised in a genetically identical animal by using the identical preparation of antigen and the same immunization protocol. Second, antisera can be produced in only limited volumes, and thus it is impossible to use the identical serological reagent in a long or complex series of experiments or clinical tests. Finally, even antibodies purified by affinity chromatography (see Section A-5) may include minor populations of antibodies that give unexpected cross-reactions, which confound the analysis of experiments. To avoid these problems, and to harness the full potential of antibodies, it was necessary to develop a way of making an unlimited supply of antibody molecules of homogeneous structure and known specificity. This has been achieved through the production of monoclonal antibodies from cultures of hybrid antibody-forming cells or, more recently, by genetic engineering.

Biochemists in search of a homogeneous preparation of antibody that they could subject to detailed chemical analysis turned early to proteins produced by patients with multiple myeloma, a common tumor of plasma cells. It was known that antibodies are normally produced by plasma cells and since this disease is associated with the presence of large amounts of a homogeneous gamma globulin called a **myeloma protein** in the patient's serum, it seemed likely that myeloma proteins would serve as models for normal antibody molecules. Thus, much of the early knowledge of antibody structure came from studies on myeloma proteins. These studies showed that monoclonal antibodies could be obtained from immortalized plasma cells. However, the antigen specificity of most myeloma proteins was unknown, which limited their usefulness as objects of study or as immunological tools.

This problem was solved by Georges Köhler and Cesar Milstein, who devised a technique for producing a homogeneous population of antibodies of known antigenic specificity. They did this by fusing spleen cells from an immunized mouse to cells of a mouse myeloma to produce hybrid cells that both proliferated indefinitely and secreted antibody specific for the antigen used to immunize the spleen cell donor. The spleen cell provides the ability to make specific antibody, while the myeloma cell provides the ability to grow indefinitely in culture and secrete immunoglobulin continuously. By using a myeloma cell partner that produces no antibody proteins itself, the antibody produced by the hybrid cells comes only from the immune spleen cell partner. After fusion, the hybrid cells are selected using drugs that kill the myeloma parental cell, while the unfused parental spleen cells have a limited life-span and soon die, so that only hybrid myeloma cell lines or **hybridomas** survive. Those hybridomas producing antibody of the desired specificity are then identified and cloned by regrowing the cultures from single cells (Fig. A.14). Since each hybridoma is a **clone** derived from fusion with a single B cell, all the antibody molecules it produces are identical in structure, including their antigen-binding site and isotype. Such antibodies are called **monoclonal antibodies**. This technology has revolutionized the use of antibodies by providing a limitless supply of antibody of a single and known specificity. Monoclonal antibodies are now used in most serological assays, as diagnostic probes, and as therapeutic agents. So far, however, only mouse monoclonals are routinely produced and efforts to use this same approach to make human monoclonal antibodies have met with very limited success.

A-13 Phage display libraries for antibody V-region production.

This is a technique for producing antibody-like molecules. Gene segments encoding the antigen-binding variable or V domains of antibodies are fused to genes encoding the coat protein of a bacteriophage. Bacteriophage containing such gene fusions are used to infect bacteria, and the resulting phage particles have coats that express the antibody-like fusion protein, with

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HIGHLIGHTS OF PRESCRIBING INFORMATION
These highlights do not include all the information needed to use SOLIRIS safely and effectively. See full prescribing information for SOLIRIS.

SOLIRIS® (eculizumab) injection, for intravenous use
Initial U.S. Approval: 2007

WARNING: SERIOUS MENINGOCOCCAL INFECTIONS
See full prescribing information for complete boxed warning

Life-threatening and fatal meningococcal infections have occurred in patients treated with Soliris and may become rapidly life-threatening or fatal if not recognized and treated early (5.1).

- **Comply with the most current Advisory Committee on Immunization Practices (ACIP) recommendations for meningococcal vaccination in patients with complement deficiencies (5.1).**
- **Immunize patients with meningococcal vaccines at least 2 weeks prior to administering the first dose of Soliris, unless the risks of delaying Soliris therapy outweigh the risks of developing a meningococcal infection. (See *Warnings and Precautions* (5.1) for additional guidance on the management of the risk of meningococcal infection.)**
- **Vaccination reduces, but does not eliminate, the risk of meningococcal infections. Monitor patients for early signs of meningococcal infections, and evaluate immediately if infection is suspected.**

Soliris is available only through a restricted program under a Risk Evaluation and Mitigation Strategy (REMS). Under the Soliris REMS, prescribers must enroll in the program (5.1).

RECENT MAJOR CHANGES

Indications and Usage (1.4)	06/2019
Dosage and Administration (2.4, 2.5)	06/2019
Dosage and Administration (2.5, 2.6, 2.7)	07/2018
Warnings and Precautions (5.1, 5.2)	07/2018

INDICATIONS AND USAGE

Soliris is a complement inhibitor indicated for:

- The treatment of patients with paroxysmal nocturnal hemoglobinuria (PNH) to reduce hemolysis (1.1).
- The treatment of patients with atypical hemolytic uremic syndrome (aHUS) to inhibit complement-mediated thrombotic microangiopathy (1.2).

  Limitation of Use
  Soliris is not indicated for the treatment of patients with Shiga toxin E. coli related hemolytic uremic syndrome (STEC-HUS).
- The treatment of generalized myasthenia gravis (gMG) in adult patients who are anti-acetylcholine receptor (AchR) antibody positive (1.3).
- The treatment of neuromyelitis optica spectrum disorder (NMOSD) in adult patients who are anti-aquaporin-4 (AQP4) antibody positive (1.4).

DOSAGE AND ADMINISTRATION

For intravenous infusion only
PNH Dosage Regimen: (2.2)
aHUS Dosage Regimen: (2.3)
gMG and NMOSD Dosage Regimen: (2.4)

DOSAGE FORMS AND STRENGTHS

Injection: 300 mg/30 mL (10 mg/mL) in a single-dose vial (3).

CONTRAINDICATIONS

Soliris is contraindicated in:

- Patients with unresolved serious *Neisseria meningitidis* infection (4).
- Patients who are not currently vaccinated against *Neisseria meningitidis*, unless the risks of delaying Soliris treatment outweigh the risks of developing a meningococcal infection (5.1).

WARNINGS AND PRECAUTIONS

- Discontinue Soliris in patients who are being treated for serious meningococcal infections (5.1).
- Use caution when administering Soliris to patients with any other systemic infection (5.2).

ADVERSE REACTIONS

The most frequently reported adverse reactions in the PNH randomized trial (≥10% overall and greater than placebo) are: headache, nasopharyngitis, back pain, and nausea (6.1).

The most frequently reported adverse reactions in aHUS single arm prospective trials (≥20%) are: headache, diarrhea, hypertension, upper respiratory infection, abdominal pain, vomiting, nasopharyngitis, anemia, cough, peripheral edema, nausea, urinary tract infections, pyrexia (6.1).

The most frequently reported adverse reaction in the gMG placebo-controlled clinical trial (≥10%) is: musculoskeletal pain (6.1).

The most frequently reported adverse reactions in the NMOSD placebo-controlled trial (≥10%) are: upper respiratory infection, nasopharyngitis, diarrhea, back pain, dizziness, influenza, arthralgia, pharyngitis, and contusion (6.1)

To report SUSPECTED ADVERSE REACTIONS, contact Alexion Pharmaceuticals, Inc. at 1-844-259-6783 or FDA at 1-800-FDA-1088 or www.fda.gov/medwatch.

See 17 PATIENT COUNSELING INFORMATION and Medication Guide.

Revised: 06/2019

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FULL PRESCRIBING INFORMATION

WARNING: SERIOUS MENINGOCOCCAL INFECTIONS

Life-threatening and fatal meningococcal infections have occurred in patients treated with Soliris. Meningococcal infection may become rapidly life-threatening or fatal if not recognized and treated early [*see Warnings and Precautions (5.1)*].

- **Comply with the most current Advisory Committee on Immunization Practices (ACIP) recommendations for meningococcal vaccination in patients with complement deficiencies.**
- **Immunize patients with meningococcal vaccines at least 2 weeks prior to administering the first dose of Soliris, unless the risks of delaying Soliris therapy outweigh the risk of developing a meningococcal infection. [See *Warnings and Precautions (5.1)* for additional guidance on the management of the risk of meningococcal infection].**
- **Vaccination reduces, but does not eliminate, the risk of meningococcal infections. Monitor patients for early signs of meningococcal infections and evaluate immediately if infection is suspected.**

Soliris is available only through a restricted program under a Risk Evaluation and Mitigation Strategy (REMS). Under the Soliris REMS, prescribers must enroll in the program [*see Warnings and Precautions (5.1)*]. Enrollment in the Soliris REMS program and additional information are available by telephone: 1-888-SOLIRIS (1-888-765-4747) or at www.solirisrems.com.

1 INDICATIONS AND USAGE

1.1 Paroxysmal Nocturnal Hemoglobinuria (PNH)

Soliris is indicated for the treatment of patients with paroxysmal nocturnal hemoglobinuria (PNH) to reduce hemolysis.

1.2 Atypical Hemolytic Uremic Syndrome (aHUS)

Soliris is indicated for the treatment of patients with atypical hemolytic uremic syndrome (aHUS) to inhibit complement-mediated thrombotic microangiopathy.

Limitation of Use

Soliris is not indicated for the treatment of patients with Shiga toxin E. coli related hemolytic uremic syndrome (STEC-HUS).

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1.3 Generalized Myasthenia Gravis (gMG)

Soliris is indicated for the treatment of generalized myasthenia gravis (gMG) in adult patients who are anti-acetylcholine receptor (AchR) antibody positive.

1.4 Neuromyelitis Optica Spectrum Disorder (NMOSD)

Soliris is indicated for the treatment of neuromyelitis optica spectrum disorder (NMOSD) in adult patients who are anti-aquaporin-4 (AQP4) antibody positive.

2 DOSAGE AND ADMINISTRATION

2.1 Recommended Vaccination and Prophylaxis

Vaccinate patients according to current ACIP guidelines to reduce the risk of serious infection [*see Warnings and Precautions (5.1 and 5.2)*].

Provide two weeks of antibacterial drug prophylaxis to patients if Soliris must be initiated immediately and vaccines are administered less than two weeks before starting Soliris therapy.

Healthcare professionals who prescribe Soliris must enroll in the Soliris REMS [*see Warnings and Precautions (5.1)*].

2.2 Recommended Dosage Regimen □PNH

For patients 18 years of age and older, Soliris therapy consists of:

- 600 mg weekly for the first 4 weeks, followed by
- 900 mg for the fifth dose 1 week later, then
- 900 mg every 2 weeks thereafter.

Administer Soliris at the recommended dosage regimen time points, or within two days of these time points *[see Warnings and Precautions (5.4)*].

2.3 Recommended Dosage Regimen □aHUS

For patients 18 years of age and older, Soliris therapy consists of:

- 900 mg weekly for the first 4 weeks, followed by
- 1200 mg for the fifth dose 1 week later, then
- 1200 mg every 2 weeks thereafter.

For patients less than 18 years of age, administer Soliris based upon body weight, according to the following schedule (Table 1):

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Table 1: Dosing Recommendations in aHUS Patients Less Than 18 Years of Age

Patient Body Weight	Induction	Maintenance
40 kg and over	900 mg weekly x 4 doses	1200 mg at week 5; then 1200 mg every 2 weeks
30 kg to less than 40 kg	600 mg weekly x 2 doses	900 mg at week 3; then 900 mg every 2 weeks
20 kg to less than 30 kg	600 mg weekly x 2 doses	600 mg at week 3; then 600 mg every 2 weeks
10 kg to less than 20 kg	600 mg weekly x 1 dose	300 mg at week 2; then 300 mg every 2 weeks
5 kg to less than 10 kg	300 mg weekly x 1 dose	300 mg at week 2; then 300 mg every 3 weeks

Administer Soliris at the recommended dosage regimen time points, or within two days of these time points.

2.4 Recommended Dosage Regimen □gMG and NMOSD

For adult patients with generalized myasthenia gravis or neuromyelitis optica spectrum disorder, Soliris therapy consists of:

- 900 mg weekly for the first 4 weeks, followed by
- 1200 mg for the fifth dose 1 week later, then
- 1200 mg every 2 weeks thereafter.

Administer Soliris at the recommended dosage regimen time points, or within two days of these time points.

2.5 Dose Adjustment in Case of Plasmapheresis, Plasma Exchange, or Fresh Frozen Plasma Infusion

For adult and pediatric patients with aHUS, and adult patients with gMG or NMOSD, supplemental dosing of Soliris is required in the setting of concomitant plasmapheresis or plasma exchange, or fresh frozen plasma infusion (PE/PI) (Table 2).

Table 2: Supplemental Dose of Soliris after PE/PI

Type of Plasma Intervention	Most Recent Soliris Dose	Supplemental Soliris Dose with Each Plasma Intervention	Timing of Supplemental Soliris Dose
Plasmapheresis or plasma exchange	300 mg	300 mg per each plasmapheresis or	Within 60 minutes after each

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		plasma exchange session	plasmapheresis or plasma exchange
	≥600 mg	600 mg per each plasmapheresis or plasma exchange session	
Fresh frozen plasma infusion	≥300 mg	300 mg per infusion of fresh frozen plasma	60 minutes prior to each infusion of fresh frozen plasma

2.6 Preparation

Dilute Soliris to a final admixture concentration of 5 mg/mL using the following steps:

- Withdraw the required amount of Soliris from the vial into a sterile syringe.
- Transfer the recommended dose to an infusion bag.
- Dilute Soliris to a final concentration of 5 mg/mL by adding the appropriate amount (equal volume of diluent to drug volume) of 0.9% Sodium Chloride Injection, USP; 0.45% Sodium Chloride Injection, USP; 5% Dextrose in Water Injection, USP; or Ringer' s Injection, USP to the infusion bag.

The final admixed Soliris 5 mg/mL infusion volume is 60 mL for 300 mg doses, 120 mL for 600 mg doses, 180 mL for 900 mg doses or 240 mL for 1200 mg doses (Table 3).

Table 3: Preparation and Reconstitution of Soliris

Soliris Dose	Diluent Volume	Final Volume
300 mg	30 mL	60 mL
600 mg	60 mL	120 mL
900 mg	90 mL	180 mL
1200 mg	120 mL	240 mL

Gently invert the infusion bag containing the diluted Soliris solution to ensure thorough mixing of the product and diluent. Discard any unused portion left in a vial, as the product contains no preservatives.

Prior to administration, the admixture should be allowed to adjust to room temperature [18°-25° C, 64°-77° F]. The admixture must not be heated in a microwave or with any heat source other than ambient air temperature.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

2.7 Administration

Only administer as an intravenous infusion.

Do not administer as an intravenous push or bolus injection.

Administer the Soliris admixture by intravenous infusion over 35 minutes in adults and 1 to 4 hours in pediatric patients via gravity feed, a syringe-type pump, or an infusion

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pump. Admixed solutions of Soliris are stable for 24 h at 2°-8° C (36°-46° F) and at room temperature.

If an adverse reaction occurs during the administration of Soliris, the infusion may be slowed or stopped at the discretion of the physician. If the infusion is slowed, the total infusion time should not exceed two hours in adults. Monitor the patient for at least one hour following completion of the infusion for signs or symptoms of an infusion reaction.

3 DOSAGE FORMS AND STRENGTHS

Injection: 300 mg/30 mL (10 mg/mL) as a clear, colorless solution in a single-dose vial.

4 CONTRAINDICATIONS

Soliris is contraindicated in:

- Patients with unresolved serious *Neisseria meningitidis* infection [*see Warnings and Precautions (5.1)*].
- Patients who are not currently vaccinated against *Neisseria meningitidis*, unless the risks of delaying Soliris treatment outweigh the risks of developing a meningococcal infection [*see Warnings and Precautions (5.1)*].

5 WARNINGS AND PRECAUTIONS

5.1 Serious Meningococcal Infections

Risk and Prevention

Life-threatening and fatal meningococcal infections have occurred in patients treated with Soliris. The use of Soliris increases a patient's susceptibility to serious meningococcal infections (septicemia and/or meningitis). Soliris is associated with an approximate 2,000-fold increased risk of meningococcal disease in comparison to the general U.S. population annual rate (0.14 per 100,000 population in 2015).

Vaccinate for meningococcal disease according to the most current Advisory Committee on Immunization Practices (ACIP) recommendations for patients with complement deficiencies. Revaccinate patients in accordance with ACIP recommendations, considering the duration of Soliris therapy.

Immunize patients without a history of meningococcal vaccination at least 2 weeks prior to receiving the first dose of Soliris. If urgent Soliris therapy is indicated in an unvaccinated patient, administer meningococcal vaccine(s) as soon as possible and provide patients with two weeks of antibacterial drug prophylaxis.

In prospective clinical studies, 75/100 patients with aHUS were treated with Soliris less than 2 weeks after meningococcal vaccination and 64 of these 75 patients received antibiotics for prophylaxis of meningococcal infection until at least 2 weeks after

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meningococcal vaccination. The benefits and risks of antibiotic prophylaxis for prevention of meningococcal infections in patients receiving Soliris have not been established.

Vaccination reduces, but does not eliminate, the risk of meningococcal infections. In clinical studies, 2 out of 196 PNH patients developed serious meningococcal infections while receiving treatment with Soliris; both had been vaccinated [*see Adverse Reactions (6.1)*]. In clinical studies among non-PNH patients, meningococcal meningitis occurred in one unvaccinated patient. In addition, 3 out of 130 previously vaccinated patients with aHUS developed meningococcal infections while receiving treatment with Soliris [*see Adverse Reactions (6.1)*].

Closely monitor patients for early signs and symptoms of meningococcal infection and evaluate patients immediately if an infection is suspected. Meningococcal infection may become rapidly life-threatening or fatal if not recognized and treated early. Discontinue Soliris in patients who are undergoing treatment for serious meningococcal infections.

REMS

Because of the risk of meningococcal infections, Soliris is available only through a restricted program under a Risk Evaluation and Mitigation Strategy (REMS). Under the Soliris REMS, prescribers must enroll in the program.

Prescribers must counsel patients about the risk of meningococcal infection, provide the patients with the REMS educational materials, and ensure patients are vaccinated with meningococcal vaccine(s).

Enrollment in the Soliris REMS program and additional information are available by telephone: 1-888-SOLIRIS (1-888-765-4747) or at www.solirisrems.com.

5.2 Other Infections

Serious infections with *Neisseria* species (other than *N. meningitidis*), including disseminated gonococcal infections, have been reported.

Soliris blocks terminal complement activation; therefore patients may have increased susceptibility to infections, especially with encapsulated bacteria. Additionally, *Aspergillus* infections have occurred in immunocompromised and neutropenic patients. Children treated with Soliris may be at increased risk of developing serious infections due to *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (Hib). Administer vaccinations for the prevention of *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (Hib) infections according to ACIP guidelines. Use caution when administering Soliris to patients with any systemic infection [*see Warnings and Precautions (5.1)*].

5.3 Monitoring Disease Manifestations after Soliris Discontinuation

Treatment Discontinuation for PNH

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Monitor patients after discontinuing Soliris for at least 8 weeks to detect hemolysis.

Treatment Discontinuation for aHUS

After discontinuing Soliris, monitor patients with aHUS for signs and symptoms of thrombotic microangiopathy (TMA) complications for at least 12 weeks. In aHUS clinical trials, 18 patients (5 in the prospective studies) discontinued Soliris treatment. TMA complications occurred following a missed dose in 5 patients, and Soliris was reinitiated in 4 of these 5 patients.

Clinical signs and symptoms of TMA include changes in mental status, seizures, angina, dyspnea, or thrombosis. In addition, the following changes in laboratory parameters may identify a TMA complication: occurrence of two, or repeated measurement of any one of the following: a decrease in platelet count by 25% or more compared to baseline or the peak platelet count during Soliris treatment; an increase in serum creatinine by 25% or more compared to baseline or nadir during Soliris treatment; or, an increase in serum LDH by 25% or more over baseline or nadir during Soliris treatment.

If TMA complications occur after Soliris discontinuation, consider reinstitution of Soliris treatment, plasma therapy [plasmapheresis, plasma exchange, or fresh frozen plasma infusion (PE/PI)], or appropriate organ-specific supportive measures.

5.4 Thrombosis Prevention and Management

The effect of withdrawal of anticoagulant therapy during Soliris treatment has not been established. Therefore, treatment with Soliris should not alter anticoagulant management.

5.5 Infusion Reactions

Administration of Soliris may result in infusion reactions, including anaphylaxis or other hypersensitivity reactions. In clinical trials, no patients experienced an infusion reaction which required discontinuation of Soliris. Interrupt Soliris infusion and institute appropriate supportive measures if signs of cardiovascular instability or respiratory compromise occur.

6 ADVERSE REACTIONS

The following serious adverse reactions are discussed in greater detail in other sections of the labeling:

- Serious Meningococcal Infections [*see Warnings and Precautions (5.1)*]
- Other Infections [*see Warnings and Precautions (5.2)*]
- Monitoring Disease Manifestations after Soliris Discontinuation [*see Warnings and Precautions (5.3)*]
- Thrombosis Prevention and Management [*see Warnings and Precautions (5.4)*]

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- Infusion Reactions [*see Warnings and Precautions (5.5)*]

6.1 Clinical Trial Experience

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice.

Meningococcal infections are the most important adverse reactions experienced by patients receiving Soliris. In PNH clinical studies, two patients experienced meningococcal sepsis. Both patients had previously received a meningococcal vaccine. In clinical studies among patients without PNH, meningococcal meningitis occurred in one unvaccinated patient. Meningococcal sepsis occurred in one previously vaccinated patient enrolled in the retrospective aHUS study during the post-study follow-up period [*see Warnings and Precautions (5.1)*].

PNH

The data described below reflect exposure to Soliris in 196 adult patients with PNH, age 18-85, of whom 55% were female. All had signs or symptoms of intravascular hemolysis. Soliris was studied in a placebo-controlled clinical study (PNH Study 1, in which 43 patients received Soliris and 44, placebo); a single arm clinical study (PNH Study 2); and a long term extension study (E05-001). 182 patients were exposed for greater than one year. All patients received the recommended Soliris dose regimen.

Table 4 summarizes the adverse reactions that occurred at a numerically higher rate in the Soliris group than the placebo group and at a rate of 5% or more among patients treated with Soliris.

Table 4: Adverse Reactions Reported in 5% or More of Soliris Treated Patients with PNH and Greater than Placebo in the Controlled Clinical Study

Reaction	Soliris (N=43) N (%)	Placebo (N=44) N (%)
Headache	19 (44)	12 (27)
Nasopharyngitis	10 (23)	8 (18)
Back pain	8 (19)	4 (9)
Nausea	7 (16)	5 (11)
Fatigue	5 (12)	1 (2)
Cough	5 (12)	4 (9)
Herpes simplex infections	3 (7)	0
Sinusitis	3 (7)	0
Respiratory tract infection	3 (7)	1 (2)
Constipation	3 (7)	2 (5)
Myalgia	3 (7)	1 (2)

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Pain in extremity	3 (7)	1 (2)
Influenza-like illness	2 (5)	1 (2)

In the placebo-controlled clinical study, serious adverse reactions occurred among 4 (9%) patients receiving Soliris and 9 (21%) patients receiving placebo. The serious reactions included infections and progression of PNH. No deaths occurred in the study and no patients receiving Soliris experienced a thrombotic event; one thrombotic event occurred in a patient receiving placebo.

Among 193 patients with PNH treated with Soliris in the single arm, clinical study or the follow-up study, the adverse reactions were similar to those reported in the placebo-controlled clinical study. Serious adverse reactions occurred among 16% of the patients in these studies. The most common serious adverse reactions were: viral infection (2%), headache (2%), anemia (2%), and pyrexia (2%).

aHUS

The safety of Soliris therapy in patients with aHUS was evaluated in four prospective, single-arm studies, three in adult and adolescent patients (Studies C08-002A/B, C08-003A/B, and C10-004), one in pediatric and adolescent patients (Study C10-003), and one retrospective study (Study C09-001r).

The data described below were derived from 78 adult and adolescent patients with aHUS in Studies C08-002A/B, C08-003A/B and C10-004. All patients received the recommended dosage of Soliris. Median exposure was 67 weeks (range: 2-145 weeks). Table 5 summarizes all adverse events reported in at least 10% of patients in Studies C08-002A/B, C08-003A/B and C10-004 combined.

Table 5: Per Patient Incidence of Adverse Events in 10% or More Adult and Adolescent Patients Enrolled in Studies C08-002A/B, C08-003A/B and C10-004 Separately and in Total

	Number (%) of Patients			
	C08-002A/B (N=17)	**C08-003A/B (N=20)**	**C10-004 (N=41)**	**Total (N=78)**
Vascular Disorders				
Hypertension[a]	10 (59)	9 (45)	7 (17)	26 (33)
Hypotension	2 (12)	4 (20)	7 (17)	13 (17)
Infections and Infestations				
Bronchitis	3 (18)	2 (10)	4 (10)	9 (12)
Nasopharyngitis	3 (18)	11 (55)	7 (17)	21 (27)
Gastroenteritis	3 (18)	4 (20)	2 (5)	9 (12)
Upper respiratory tract infection	5 (29)	8 (40)	2 (5)	15 (19)
Urinary tract infection	6 (35)	3 (15)	8 (20)	17 (22)
Gastrointestinal Disorders				

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	Number (%) of Patients			
	C08-002A/B (N=17)	C08-003A/B (N=20)	C10-004 (N=41)	Total (N=78)
Diarrhea	8 (47)	8 (40)	12 (32)	29 (37)
Vomiting	8 (47)	9 (45)	6 (15)	23 (30)
Nausea	5 (29)	8 (40)	5 (12)	18 (23)
Abdominal pain	3 (18)	6 (30)	6 (15)	15 (19)
Nervous System Disorders				
Headache	7 (41)	10 (50)	15 (37)	32 (41)
Blood and Lymphatic System Disorders				
Anemia	6 (35)	7 (35)	7 (17)	20 (26)
Leukopenia	4 (24)	3 (15)	5 (12)	12 (15)
Psychiatric Disorders				
Insomnia	4 (24)	2 (10)	5 (12)	11 (14)
Renal and Urinary Disorders				
Renal Impairment	5 (29)	3 (15)	6 (15)	14 (18)
Proteinuria	2 (12)	1 (5)	5 (12)	8 (10)
Respiratory, Thoracic and Mediastinal Disorders				
Cough	4 (24)	6 (30)	8 (20)	18 (23)
General Disorders and Administration Site Conditions				
Fatigue	3 (18)	4 (20)	3 (7)	10 (13)
Peripheral edema	5 (29)	4 (20)	9 (22)	18 (23)
Pyrexia	4 (24)	5 (25)	7 (17)	16 (21)
Asthenia	3 (18)	4 (20)	6 (15)	13 (17)
Eye Disorder	5 (29)	2 (10)	8 (20)	15 (19)
Metabolism and Nutrition Disorders				
Hypokalemia	3 (18)	2 (10)	4 (10)	9 (12)
Neoplasms benign, malignant, and unspecified (including cysts and polyps)	1 (6)	6 (30)	1 (20)	8 (10)
Skin and Subcutaneous Tissue Disorders				
Rash	2 (12)	3 (15)	6 (15)	11 (14)
Pruritus	1 (6)	3 (15)	4 (10)	8 (10)
Musculoskeletal and Connective Tissue Disorders				
Arthralgia	1 (6)	2 (10)	7 (17)	10 (13)
Back pain	3 (18)	3 (15)	2 (5)	8 (10)

[a.] includes the preferred terms hypertension, accelerated hypertension, and malignant hypertension.

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In Studies C08-002A/B, C08-003A/B and C10-004 combined, 60% (47/78) of patients experienced a serious adverse event (SAE). The most commonly reported SAEs were infections (24%), hypertension (5%), chronic renal failure (5%), and renal impairment (5%). Five patients discontinued Soliris due to adverse events; three due to worsening renal function, one due to new diagnosis of Systemic Lupus Erythematosus, and one due to meningococcal meningitis.

Study C10-003 included 22 pediatric and adolescent patients, of which 18 patients were less than 12 years of age. All patients received the recommended dosage of Soliris. Median exposure was 44 weeks (range: 1 dose-87 weeks).

Table 6 summarizes all adverse events reported in at least 10% of patients enrolled in Study C10-003.

Table 6: Per Patient Incidence of Adverse Reactions in 10% or More Patients Enrolled in Study C10-003

	1 month to <12 yrs (N=18)	Total (N=22)
Eye Disorders	3 (17)	3 (14)
Gastrointestinal Disorders		
Abdominal pain	6 (33)	7 (32)
Diarrhea	5 (28)	7 (32)
Vomiting	4 (22)	6 (27)
Dyspepsia	0	3 (14)
General Disorders and Administration Site Conditions		
Pyrexia	9 (50)	11 (50)
Infections and Infestations		
Upper respiratory tract infection	5 (28)	7 (32)
Nasopharyngitis	3 (17)	6 (27)
Rhinitis	4 (22)	4 (18)
Urinary Tract infection	3 (17)	4 (18)
Catheter site infection	3 (17)	3 (14)
Musculoskeletal and Connective Tissue Disorders		
Muscle spasms	2 (11)	3 (14)
Nervous System Disorders		
Headache	3 (17)	4 (18)
Renal and Urinary Disorders	3 (17)	4 (18)
Respiratory, Thoracic and Mediastinal Disorders		
Cough	7 (39)	8 (36)
Oropharyngeal pain	1 (6)	3 (14)

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	1 month to <12 yrs (N=18)	Total (N=22)
Skin and Subcutaneous Tissue Disorders		
Rash	4 (22)	4 (18)
Vascular Disorders		
Hypertension	4 (22)	4 (18)

In Study C10-003, 59% (13/22) of patients experienced a serious adverse event (SAE). The most commonly reported SAEs were hypertension (9%), viral gastroenteritis (9%), pyrexia (9%), and upper respiratory infection (9%). One patient discontinued Soliris due to an adverse event (severe agitation).

Analysis of retrospectively collected adverse event data from pediatric and adult patients enrolled in Study C09-001r (N=30) revealed a safety profile that was similar to that which was observed in the two prospective studies. Study C09-001r included 19 pediatric patients less than 18 years of age. Overall, the safety of Soliris in pediatric patients with aHUS enrolled in Study C09-001r appeared similar to that observed in adult patients. The most common (≥15%) adverse events occurring in pediatric patients are presented in Table 7.

Table 7: Adverse Reactions Occurring in at Least 15% of Patients Less than 18 Years of Age Enrolled in Study C09-001r

	Number (%) of Patients			
	< 2 yrs (N=5)	2 to < 12 yrs (N=10)	12 to <18 yrs (N=4)	Total (N=19)
General Disorders and Administration Site Conditions				
Pyrexia	4 (80)	4 (40)	1 (25)	9 (47)
Gastrointestinal Disorders				
Diarrhea	1 (20)	4 (40)	1 (25)	6 (32)
Vomiting	2 (40)	1 (10)	1 (25)	4 (21)
Infections and Infestations				
Upper respiratory tract infection[a]	2 (40)	3 (30)	1 (25)	6 (32)
Respiratory, Thoracic and Mediastinal Disorders				
Cough	3 (60)	2 (20)	0 (0)	5 (26)
Nasal congestion	2 (40)	2 (20)	0 (0)	4 (21)
Cardiac Disorders				

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	Number (%) of Patients			
	< 2 yrs (N=5)	2 to < 12 yrs (N=10)	12 to <18 yrs (N=4)	Total (N=19)
Tachycardia	2 (40)	2 (20)	0 (0)	4 (21)

[a.] includes the preferred terms upper respiratory tract infection and nasopharyngitis.

Generalized Myasthenia Gravis (gMG)

In a 26-week placebo-controlled trial evaluating the effect of Soliris for the treatment of gMG (gMG Study 1), 62 patients received Soliris at the recommended dosage regimen and 63 patients received placebo [*see Clinical Studies (14.3)*]. Patients were 19 to 79 years of age, and 66% were female. Table 8 displays the most common adverse reactions from gMG Study 1 that occurred in ≥5% of Soliris-treated patients and at a greater frequency than on placebo.

Table 8: Adverse Reactions Reported in 5% or More of Soliris-Treated Patients in gMG Study 1 and at a Greater Frequency than in Placebo-Treated Patients

	Soliris (N=62) N (%)	Placebo (N=63) N (%)
Gastrointestinal Disorders		
Abdominal pain	5 (8)	3 (5)
General Disorders and Administration Site Conditions		
Peripheral edema	5 (8)	3 (5)
Pyrexia	4 (7)	2 (3)
Infections and Infestations		
Herpes simplex virus infections	5 (8)	1 (2)

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Injury, Poisoning, and Procedural Complications		
Contusion	5 (8)	2(3)
Musculoskeletal and Connective Tissue Disorders		
Musculoskeletal pain	9 (15)	5 (8)

The most common adverse reactions (≥10%) that occurred in Soliris-treated patients in the long-term extension to gMG Study 1, Study ECU-MG-302, and that are not included in Table 8 were headache (26%), nasopharyngitis (24%), diarrhea (15%), arthralgia (12%), upper respiratory tract infection (11%), and nausea (10%).

Neuromyelitis Optica Spectrum Disorder (NMOSD)

In a placebo-controlled trial evaluating the effect of Soliris for the treatment of NMOSD (NMOSD Study 1), 96 patients received Soliris at the recommended dosage regimen and 47 patients received placebo [*see Clinical Studies (14.4)*]. Patients were 19 to 75 years of age (mean 44 years of age), and 91% were female. Table 9 displays the most common adverse reactions from NMOSD Study 1 that occurred in ≥5% of Soliris-treated patients and at a greater frequency than on placebo.

Table 9: Adverse Reactions Reported in 5% or More of Soliris-Treated Patients in NMOSD Study 1 and at a Greater Frequency than in Placebo-Treated Patients

	Soliris (N=96) N (%)	**Placebo (N=47) N (%)**
Events/Patients	1295/88	617/45
Blood and lymphatic system disorders		
Leukopenia	5 (5)	1 (2)
Lymphopenia	5 (5)	0 (0)
Eye disorders		
Cataract	6 (6)	2 (4)

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	Soliris (N=96) N (%)	Placebo (N=47) N (%)
Gastrointestinal disorders		
Diarrhea	15 (16)	7 (15)
Constipation	9 (9)	3 (6)
General disorders and administration site conditions		
Asthenia	5 (5)	1 (2)
Infections and infestations		
Upper respiratory tract infection	28 (29)	6 (13)
Nasopharyngitis	20 (21)	9 (19)
Influenza	11 (11)	2 (4)
Pharyngitis	10 (10)	3 (6)
Bronchitis	9 (9)	3 (6)
Conjunctivitis	9 (9)	4 (9)
Cystitis	8 (8)	1 (2)
Hordeolum	7 (7)	0 (0)
Sinusitis	6 (6)	0 (0)
Cellulitis	5 (5)	1 (2)
Injury, poisoning and procedural complications		
Contusion	10 (10)	2 (4)
Metabolism and nutrition disorders		
Decreased appetite	5 (5)	1 (2)
Musculoskeletal and connective tissue disorders		
Back pain	14 (15)	6 (13)

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	Soliris (N=96) N (%)	Placebo (N=47) N (%)
Arthralgia	11 (11)	5 (11)
Musculoskeletal pain	6 (6)	0 (0)
Muscle spasms	5 (5)	2 (4)
Nervous system disorders		
Dizziness	14 (15)	6 (13)
Paraesthesia	8 (8)	3 (6)
Respiratory, thoracic and mediastinal disorders		
Oropharyngeal pain	7 (7)	2 (4)
Skin and subcutaneous tissue disorders		
Alopecia	5 (5)	2 (4)

6.2 Immunogenicity

As with all proteins, there is a potential for immunogenicity. The detection of antibody formation is highly dependent on the sensitivity and specificity of the assay. Additionally, the observed incidence of antibody (including neutralizing antibody) positivity in an assay may be influenced by several factors including assay methodology, sample handling, timing of sample collection, concomitant medications, and underlying disease. For these reasons, comparison of the incidence of antibodies to eculizumab in the studies described below with the incidence of antibodies in other studies or to other products may be misleading.

The immunogenicity of Soliris has been evaluated using two different immunoassays for the detection of anti-eculizumab antibodies: a direct enzyme-linked immunosorbent assay (ELISA) using the Fab fragment of eculizumab as target was used for the PNH indication; and an electro-chemiluminescence (ECL) bridging assay using the eculizumab whole molecule as target was used for the aHUS, gMG, and NMOSD indications, as well as for additional patients with PNH. In the PNH population, antibodies to Soliris were detected in 3/196 (2%) patients using the ELISA assay and in 5/161 (3%) patients using

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the ECL assay. In the aHUS population, antibodies to Soliris were detected in 3/100 (3%) patients using the ECL assay. None of the 62 patients with gMG had antibodies to Soliris detected following the 26-week active treatment. Two of the 96 (2%) Soliris-treated patients with NMOSD had antibodies to Soliris detected during the entire treatment period.

An ECL based neutralizing assay with a low sensitivity of 2 mcg/mL was performed to detect neutralizing antibodies for the 5 patients with PNH, the 3 patients with aHUS, and the 2 patients with NMOSD with anti-eculizumab antibody positive samples using the ECL assay. Two of 161 patients with PNH (1.2%) and 1 of 100 patients with aHUS (1%), and none of the 96 patients with NMOSD had low positive values for neutralizing antibodies.

No apparent correlation of antibody development to clinical response was observed.

6.3 Postmarketing Experience

The following adverse reactions have been identified during post-approval use of Soliris. Because these reactions are reported voluntarily from a population of uncertain size, it is not always possible to reliably estimate their frequency or establish a causal relationship to Soliris exposure.

Fatal or serious infections: *Neisseria gonorrhoeae, Neisseria meningitidis, Neisseria sicca/subflava, Neisseria spp* unspecified

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Risk Summary

Limited data on outcomes of pregnancies that have occurred following Soliris use in pregnant women have not identified a concern for specific adverse developmental outcomes (*see Data*). There are risks to the mother and fetus associated with untreated paroxysmal nocturnal hemoglobinuria (PNH) and atypical hemolytic uremic syndrome (aHUS) in pregnancy (*see Clinical Considerations*). Animal studies using a mouse analogue of the Soliris molecule (murine anti-C5 antibody) showed increased rates of developmental abnormalities and an increased rate of dead and moribund offspring at doses 2-8 times the human dose (*see Data*).

The estimated background risk of major birth defects and miscarriage for the indicated populations is unknown. All pregnancies have a background risk of birth defect, loss, or other adverse outcomes. In the U.S. general population, the estimated background risk of major birth defect and miscarriage in clinically recognized pregnancies is 2-4% and 15-20%, respectively.

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Clinical Considerations

Disease-associated maternal and/or fetal/neonatal risk

PNH in pregnancy is associated with adverse maternal outcomes, including worsening cytopenias, thrombotic events, infections, bleeding, miscarriages and increased maternal mortality, and adverse fetal outcomes, including fetal death and premature delivery.

aHUS in pregnancy is associated with adverse maternal outcomes, including pre-eclampsia and preterm delivery, and adverse fetal/neonatal outcomes, including intrauterine growth restriction (IUGR), fetal death and low birth weight.

Data

Human Data

A pooled analysis of prospectively (50.3%) and retrospectively (49.7%) collected data in more than 300 pregnant women with live births following exposure to Soliris have not suggested safety concerns. However, these data cannot definitively exclude any drug-associated risk during pregnancy, because of the limited sample size.

Animal Data

Animal reproduction studies were conducted in mice using doses of a murine anti-C5 antibody that approximated 2-4 times (low dose) and 4-8 times (high dose) the recommended human Soliris dose, based on a body weight comparison. When animal exposure to the antibody occurred in the time period from before mating until early gestation, no decrease in fertility or reproductive performance was observed. When maternal exposure to the antibody occurred during organogenesis, two cases of retinal dysplasia and one case of umbilical hernia were observed among 230 offspring born to mothers exposed to the higher antibody dose; however, the exposure did not increase fetal loss or neonatal death. When maternal exposure to the antibody occurred in the time period from implantation through weaning, a higher number of male offspring became moribund or died (1/25 controls, 2/25 low dose group, 5/25 high dose group). Surviving offspring had normal development and reproductive function.

8.2 Lactation

Risk Summary

Although limited published data does not report detectable levels of eculizumab in human milk, maternal IgG is known to be present in human milk. Available information is insufficient to inform the effect of eculizumab on the breastfed infant. There are no data on the effects of eculizumab on milk production. The developmental and health benefits of breastfeeding should be considered along with the mother's clinical need for Soliris and any potential adverse effects on the breastfed child from eculizumab or from the underlying maternal condition.

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8.4 Pediatric Use

Safety and effectiveness of Soliris for the treatment of PNH, gMG, or NMOSD in pediatric patients have not been established.

The safety and effectiveness of Soliris for the treatment of aHUS have been established in pediatric patients. Use of Soliris in pediatric patients for this indication is supported by evidence from four adequate and well-controlled clinical studies assessing the safety and effectiveness of Soliris for the treatment of aHUS. The studies included a total of 47 pediatric patients (ages 2 months to 17 years). The safety and effectiveness of Soliris for the treatment of aHUS appear similar in pediatric and adult patients [*see Adverse Reactions (6.1), and Clinical Studies (14.2)*].

Administer vaccinations for the prevention of infection due to *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (Hib) according to ACIP guidelines [*see Warnings and Precautions (5.1, 5.2)*].

8.5 Geriatric Use

Fifty-one patients 65 years of age or older (15 with PNH, 4 with aHUS, 26 with gMG, and 6 with NMOSD) were treated with Soliris in clinical trials in the approved indications. Although there were no apparent age-related differences observed in these studies, the number of patients aged 65 and over is not sufficient to determine whether they respond differently from younger patients.

11 DESCRIPTION

Eculizumab, a complement inhibitor, is a recombinant humanized monoclonal IgG2/4$_\kappa$ antibody produced by murine myeloma cell culture and purified by standard bioprocess technology. Eculizumab contains human constant regions from human IgG2 sequences and human IgG4 sequences and murine complementarity-determining regions grafted onto the human framework light- and heavy-chain variable regions. Eculizumab is composed of two 448 amino acid heavy chains and two 214 amino acid light chains and has a molecular weight of approximately 148 kDa.

Soliris (eculizumab) injection is a sterile, clear, colorless, preservative-free 10 mg/mL solution for intravenous infusion and is supplied in 30-mL single-dose vials. The product is formulated at pH 7 and each 30 mL vial contains 300 mg of eculizumab, polysorbate 80 (6.6 mg) (vegetable origin), sodium chloride (263.1 mg), sodium phosphate dibasic (53.4 mg), sodium phosphate monobasic (13.8 mg), and Water for Injection, USP.

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12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

Eculizumab, the active ingredient in Soliris, is a monoclonal antibody that specifically binds to the complement protein C5 with high affinity, thereby inhibiting its cleavage to C5a and C5b and preventing the generation of the terminal complement complex C5b-9.

Soliris inhibits terminal complement-mediated intravascular hemolysis in PNH patients and complement-mediated thrombotic microangiopathy (TMA) in patients with aHUS.

The precise mechanism by which eculizumab exerts its therapeutic effect in gMG patients is unknown, but is presumed to involve reduction of terminal complement complex C5b-9 deposition at the neuromuscular junction.

The precise mechanism by which eculizumab exerts its therapeutic effect in NMOSD is unknown, but is presumed to involve inhibition of aquaporin-4-antibody induced terminal complement C5b-9 deposition.

12.2 Pharmacodynamics

In the placebo-controlled clinical study (PNH Study 1), Soliris when administered as recommended reduced serum LDH levels from 2200 ± 1034 U/L (mean ± SD) at baseline to 700 ± 388 U/L by week one and maintained the effect through the end of the study at week 26 (327 ± 433 U/L) in patients with PNH. In the single arm clinical study (PNH Study 2), the effect was maintained through week 52 [*see Clinical Studies (14)*].

In patients with PNH, aHUS, gMG, and NMOSD, free C5 concentrations of < 0.5 mcg/mL was correlated with complete blockade of terminal complement activity.

12.3 Pharmacokinetics

Following intravenous maintenance doses of 900 mg once every 2 weeks in patients with PNH, the week 26 observed mean ± SD serum eculizumab maximum concentration (C_{max}) was 194 ± 76 mcg/mL and the trough concentration (C_{trough}) was 97 ± 60 mcg/mL. Following intravenous maintenance doses of 1200 mg once every 2 weeks in patients with aHUS, the week 26 observed mean ± SD C_{trough} was 242 ± 101 mcg/mL. Following intravenous maintenance doses of 1200 mg once every 2 weeks in patients with gMG, the week 26 observed mean ± SD C_{max} was 783 ± 288 mcg/mL and the C_{trough} was 341 ± 172 mcg/mL. Following intravenous maintenance doses of 1200 mg once every 2 weeks in patients with NMOSD, at week 24, the observed mean±SD Cmax was 877±331 and the Ctrough was 429±188 mcg/mL.

Steady state was achieved 4 weeks after starting eculizumab treatment, with accumulation ratio of approximately 2-fold in all studied indications. Population pharmacokinetic analyses showed that eculizumab pharmacokinetics were dose-linear and time-independent over the 600 mg to 1200 mg dose range, with inter-individual variability of 21% to 38%.

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Distribution

The eculizumab volume of distribution for a typical 70 kg patient was 5 L to 8 L.

Elimination

The half-life of eculizumab was approximately 270 h to 414 h.

Plasma exchange or infusion increased the clearance of eculizumab by approximately 250-fold and reduced the half-life to 1.26 h. Supplemental dosing is recommended when Soliris is administered to patients receiving plasma exchange or infusion [*see Dosage and Administration (2.5)*].

Specific Populations

Age, Sex, and Race:

The pharmacokinetics of eculizumab were not affected by age (2 months to 85 years), sex, or race.

Renal Impairment:

Renal function did not affect the pharmacokinetics of eculizumab in PNH (creatinine clearance of 8 mL/min to 396 mL/min calculated using Cockcroft-Gault formula), aHUS (estimated glomerular filtration rate [eGFR] of 5 mL/min/1.73 m^2 to105 mL/min/1.73 m^2 using the Modification of Diet in Renal Disease [MDRD] formula), or gMG patients (eGFR of 44 mL/min/1.73 m^2 to 168 mL/min/1.73 m^2 using MDRD formula).

Drug Interactions

Intravenous immunoglobulin (IVIg) treatment may interfere with the endosomal neonatal Fc receptor (FcRn) recycling mechanism of monoclonal antibodies such as eculizumab and thereby decrease serum eculizumab concentrations. Drug interaction studies have not been conducted with eculizumab in patients treated with IVIg.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

Long-term animal carcinogenicity studies of eculizumab have not been conducted.

Genotoxicity studies have not been conducted with eculizumab.

Effects of eculizumab upon fertility have not been studied in animals. Intravenous injections of male and female mice with a murine anti-C5 antibody at up to 4-8 times the equivalent of the clinical dose of Soliris had no adverse effects on mating or fertility.

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14 CLINICAL STUDIES

14.1 Paroxysmal Nocturnal Hemoglobinuria (PNH)

The safety and efficacy of Soliris in PNH patients with hemolysis were assessed in a randomized, double-blind, placebo-controlled 26 week study (PNH Study 1, NCT00122330); PNH patients were also treated with Soliris in a single arm 52 week study (PNH Study 2, NCT00122304) and in a long-term extension study (E05-001, NCT00122317). Patients received meningococcal vaccination prior to receipt of Soliris. In all studies, the dose of Soliris was 600 mg study drug every 7 ± 2 days for 4 weeks, followed by 900 mg 7 ± 2 days later, then 900 mg every 14 ± 2 days for the study duration. Soliris was administered as an intravenous infusion over 25 - 45 minutes.

PNH Study 1:

PNH patients with at least four transfusions in the prior 12 months, flow cytometric confirmation of at least 10% PNH cells and platelet counts of at least 100,000/microliter were randomized to either Soliris (n = 43) or placebo (n = 44). Prior to randomization, all patients underwent an initial observation period to confirm the need for RBC transfusion and to identify the hemoglobin concentration (the "set-point") which would define each patient' s hemoglobin stabilization and transfusion outcomes. The hemoglobin set-point was less than or equal to 9 g/dL in patients with symptoms and was less than or equal to 7 g/dL in patients without symptoms. Endpoints related to hemolysis included the numbers of patients achieving hemoglobin stabilization, the number of RBC units transfused, fatigue, and health-related quality of life. To achieve a designation of hemoglobin stabilization, a patient had to maintain a hemoglobin concentration above the hemoglobin set-point and avoid any RBC transfusion for the entire 26 week period. Hemolysis was monitored mainly by the measurement of serum LDH levels, and the proportion of PNH RBCs was monitored by flow cytometry. Patients receiving anticoagulants and systemic corticosteroids at baseline continued these medications.

Major baseline characteristics were balanced (see Table 10).

Table 10: PNH Study1 Patient Baseline Characteristics

	Study 1	
Parameter	**Placebo (N=44)**	**Soliris (N=43)**
Mean age (SD)	38 (13)	42 (16)
Gender - female (%)	29 (66)	23 (54)
History of aplastic anemia or myelodysplastic syndrome (%)	12 (27)	8 (19)
Patients with history of thrombosis (events)	8 (11)	9 (16)

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Parameter	Study 1	
	Placebo (N=44)	Soliris (N=43)
Concomitant anticoagulants (%)	20 (46)	24 (56)
Concomitant steroids/immunosuppressant treatments (%)	16 (36)	14 (33)
Packed RBC units transfused per patient in previous 12 months (median (Q1,Q3))	17 (14, 25)	18 (12, 24)
Mean Hgb level (g/dL) at setpoint (SD)	8 (1)	8 (1)
Pre-treatment LDH levels (median, U/L)	2,234	2,032
Free hemoglobin at baseline (median, mg/dL)	46	41

Patients treated with Soliris had significantly reduced ($p< 0.001$) hemolysis resulting in improvements in anemia as indicated by increased hemoglobin stabilization and reduced need for RBC transfusions compared to placebo treated patients (see Table 11). These effects were seen among patients within each of the three pre-study RBC transfusion strata (4 - 14 units; 15 - 25 units; > 25 units). After 3 weeks of Soliris treatment, patients reported less fatigue and improved health-related quality of life. Because of the study sample size and duration, the effects of Soliris on thrombotic events could not be determined.

Table 11: PNH Study 1 Results

	Placebo (N=44)	Soliris (N=43)
Percentage of patients with stabilized hemoglobin levels	0	49
Packed RBC units transfused per patient (median)	10	0
(range)	(2 - 21)	(0 - 16)
Transfusion avoidance (%)	0	51
LDH levels at end of study (median, U/L)	2,167	239
Free hemoglobin at end of study (median, mg/dL)	62	5

PNH Study 2 and Extension Study:

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PNH patients with at least one transfusion in the prior 24 months and at least 30,000 platelets/microliter received Soliris over a 52-week period. Concomitant medications included anti-thrombotic agents in 63% of the patients and systemic corticosteroids in 40% of the patients. Overall, 96 of the 97 enrolled patients completed the study (one patient died following a thrombotic event). A reduction in intravascular hemolysis as measured by serum LDH levels was sustained for the treatment period and resulted in a reduced need for RBC transfusion and less fatigue. 187 Soliris-treated PNH patients were enrolled in a long term extension study. All patients sustained a reduction in intravascular hemolysis over a total Soliris exposure time ranging from 10 to 54 months. There were fewer thrombotic events with Soliris treatment than during the same period of time prior to treatment. However, the majority of patients received concomitant anticoagulants; the effects of anticoagulant withdrawal during Soliris therapy was not studied [*see Warnings and Precautions (5.4)*].

14.2 Atypical Hemolytic Uremic Syndrome (aHUS)

Five single-arm studies [four prospective: C08-002A/B (NCT00844545 and NCT00844844), C08-003A/B (NCT00838513 and NCT00844428), C10-003 (NCT01193348), and C10-004 (NCT01194973); and one retrospective: C09-001r (NCT01770951)] evaluated the safety and efficacy of Soliris for the treatment of aHUS. Patients with aHUS received meningococcal vaccination prior to receipt of Soliris or received prophylactic treatment with antibiotics until 2 weeks after vaccination. In all studies, the dose of Soliris in adult and adolescent patients was 900 mg every 7 ± 2 days for 4 weeks, followed by 1200 mg 7 ± 2 days later, then 1200 mg every 14 ± 2 days thereafter. The dosage regimen for pediatric patients weighing less than 40 kg enrolled in Study C09-001r and Study C10-003 was based on body weight [*see Dosage and Administration (2.3)*]. Efficacy evaluations were based on thrombotic microangiopathy (TMA) endpoints.

Endpoints related to TMA included the following:

- platelet count change from baseline
- hematologic normalization *(maintenance of normal platelet counts and LDH levels for at least four weeks)*
- complete TMA response *(hematologic normalization plus at least a 25% reduction in serum creatinine for a minimum of four weeks)*
- TMA-event free status *(absence for at least 12 weeks of a decrease in platelet count of >25% from baseline, plasma exchange or plasma infusion, and new dialysis requirement)*
- Daily TMA intervention rate *(defined as the number of plasma exchange or plasma infusion interventions and the number of new dialyses required per patient per day).*

aHUS Resistant to PE/PI (Study C08-002A/B)

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Study C08-002A/B enrolled patients who displayed signs of thrombotic microangiopathy (TMA) despite receiving at least four PE/PI treatments the week prior to screening. One patient had no PE/PI the week prior to screening because of PE/PI intolerance. In order to qualify for enrollment, patients were required to have a platelet count ≤150 x 10^9/L, evidence of hemolysis such as an elevation in serum LDH, and serum creatinine above the upper limits of normal, without the need for chronic dialysis. The median patient age was 28 (range: 17 to 68 years). Patients enrolled in Study C08-002A/B were required to have ADAMTS13 activity level above 5%; observed range of values in the trial were 70%-121%. Seventy-six percent of patients had an identified complement regulatory factor mutation or auto-antibody. Table 12 summarizes the key baseline clinical and disease-related characteristics of patients enrolled in Study C08-002A/B.

Table 12: Baseline Characteristics of Patients Enrolled in Study C08-002A/B

Parameter	C08-002A/B (N=17)
Time from aHUS diagnosis until screening in months, median (min, max)	10 (0.26, 236)
Time from current clinical TMA manifestation until screening in months, median (min, max)	<1 (<1, 4)
Baseline platelet count (× 10^9/L), median (range)	118 (62, 161)
Baseline LDH (U/L), median (range)	269 (134, 634)

Patients in Study C08-002A/B received Soliris for a minimum of 26 weeks. In Study C08-002A/B, the median duration of Soliris therapy was approximately 100 weeks (range: 2 weeks to 145 weeks).

Renal function, as measured by eGFR, was improved and maintained during Soliris therapy. The mean eGFR (± SD) increased from 23 ± 15 mL/min/1.73m^2 at baseline to 56 ± 40 mL/min/1.73m^2 by 26 weeks; this effect was maintained through 2 years (56 ± 30 mL/min/1.73m^2). Four of the five patients who required dialysis at baseline were able to discontinue dialysis.

Reduction in terminal complement activity and an increase in platelet count relative to baseline were observed after commencement of Soliris. Soliris reduced signs of complement-mediated TMA activity, as shown by an increase in mean platelet counts from baseline to 26 weeks. In Study C08-002A/B, mean platelet count (± SD) increased from 109 ± 32 x10^9/L at baseline to 169 ± 72 x10^9/L by one week; this effect was maintained through 26 weeks (210 ± 68 x10^9/L), and 2 years (205 ± 46 x10^9/L). When treatment was continued for more than 26 weeks, two additional patients achieved Hematologic Normalization as well as Complete TMA response. Hematologic Normalization and Complete TMA response were maintained by all responders. In Study C08-002A/B, responses to Soliris were similar in patients with and without identified mutations in genes encoding complement regulatory factor proteins.

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Table 13 summarizes the efficacy results for Study C08-002A/B.

Table 13: Efficacy Results for Study C08-002A/B

Efficacy Parameter	Study C08-002A/B at 26 wks[1] (N=17)	Study C08-002A/B at 2 yrs[2] (N=17)
Complete TMA response, n (%) Median Duration of complete TMA response, weeks (range)	11 (65) 38 (25, 56)	13 (77) 99 (25, 139)
eGFR improvement ≥15 mL/min/1.73 m^2, n (%) Median duration of eGFR improvement, days (range)	9 (53) 251 (70, 392)	10 (59) ND
Hematologic normalization, n (%) Median Duration of hematologic normalization, weeks (range)	13 (76) 37 (25, 62)	15 (88) 99 (25, 145)
TMA event-free status, n (%)	15 (88)	15 (88)
Daily TMA intervention rate, median (range) Before eculizumab On eculizumab treatment	 0.82 (0.04, 1.52) 0 (0, 0.31)	 0.82 (0.04, 1.52) 0 (0, 0.36)

[1] At data cut-off (September 8, 2010).
[2] At data cut-off (April 20, 2012).

aHUS Sensitive to PE/PI (Study C08-003A/B)

Study C08-003A/B enrolled patients undergoing chronic PE/PI who generally did not display hematologic signs of ongoing thrombotic microangiopathy (TMA). All patients had received PT at least once every two weeks, but no more than three times per week, for a minimum of eight weeks prior to the first Soliris dose. Patients on chronic dialysis were permitted to enroll in Study C08-003A/B. The median patient age was 28 years (range: 13 to 63 years). Patients enrolled in Study C08-003A/B were required to have ADAMTS13 activity level above 5%; observed range of values in the trial were 37%-118%. Seventy percent of patients had an identified complement regulatory factor mutation or auto-antibody. Table 14 summarizes the key baseline clinical and disease-related characteristics of patients enrolled in Study C08-003A/B.

Table 14: Baseline Characteristics of Patients Enrolled in Study C08-003A/B

Parameter	Study C08-003A/B (N=20)
Time from aHUS diagnosis until screening in months, median (min, max)	48 (0.66, 286)
Time from current clinical TMA manifestation until screening in months, median (min, max)	9 (1, 45)
Baseline platelet count (× 10^9/L), median (range)	218 (105, 421)
Baseline LDH (U/L), median (range)	200 (151, 391)

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Patients in Study C08-003A/B received Soliris for a minimum of 26 weeks. In Study C08-003A/B, the median duration of Soliris therapy was approximately 114 weeks (range: 26 to 129 weeks).

Renal function, as measured by eGFR, was maintained during Soliris therapy. The mean eGFR (± SD) was 31 ± 19 mL/min/1.73m^2 at baseline, and was maintained through 26 weeks (37 ± 21 mL/min/1.73m^2) and 2 years (40 ± 18 mL/min/1.73m^2). No patient required new dialysis with Soliris.

Reduction in terminal complement activity was observed in all patients after the commencement of Soliris. Soliris reduced signs of complement-mediated TMA activity, as shown by an increase in mean platelet counts from baseline to 26 weeks. Platelet counts were maintained at normal levels despite the elimination of PE/PI. The mean platelet count (± SD) was 228 ± 78 x 10^9/L at baseline, 233 ± 69 x 10^9/L at week 26, and 224 ± 52 x 10^9/L at 2 years. When treatment was continued for more than 26 weeks, six additional patients achieved Complete TMA response. Complete TMA Response and Hematologic Normalization were maintained by all responders. In Study C08-003A/B, responses to Soliris were similar in patients with and without identified mutations in genes encoding complement regulatory factor proteins.

Table 15 summarizes the efficacy results for Study C08-003A/B.

Table 15: Efficacy Results for Study C08-003A/B

Efficacy Parameter	Study C08-003A/B at 26 wks[1] (N=20)	Study C08-003A/B at 2 yrs[2] (N=20)
Complete TMA response, n (%) Median duration of complete TMA response, weeks (range)	5 (25) 32 (12, 38)	11 (55) 68 (38, 109)
eGFR improvement ≥15 mL/min/1.73 m^2, n (%)	1 (5)	8 (40)
TMA Event-free status n (%)	16 (80)	19 (95)
Daily TMA intervention rate, median (range) Before eculizumab On eculizumab treatment	 0.23 (0.05, 1.07) 0	 0.23 (0.05, 1.07) 0 (0, 0.01)
Hematologic normalization[4], n (%) Median duration of hematologic normalization, weeks (range)[3]	18 (90) 38 (22, 52)	18 (90) 114 (33, 125)

1. At data cut-off (September 8, 2010).
2. At data cut-off (April 20, 2012).
3. Calculated at each post-dose day of measurement (excluding Days 1 to 4) using a repeated measurement ANOVA model.
4. In Study C08-003A/B, 85% of patients had normal platelet counts and 80% of patients had normal serum LDH levels at baseline, so hematologic normalization in this population reflects maintenance of normal parameters in the absence of PE/PI.

Retrospective Study in Patients with aHUS (C09-001r)

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The efficacy results for the aHUS retrospective study (Study C09-001r) were generally consistent with results of the two prospective studies. Soliris reduced signs of complement-mediated TMA activity, as shown by an increase in mean platelet counts from baseline. Mean platelet count (± SD) increased from 171 ± 83 $x10^9$/L at baseline to 233 ±109 $x10^9$/L after one week of therapy; this effect was maintained through 26 weeks (mean platelet count (± SD) at week 26: 254 ± 79 $x10^9$/L).

A total of 19 pediatric patients (ages 2 months to 17 years) received Soliris in Study C09-001r. The median duration of Soliris therapy was 16 weeks (range 4 to 70 weeks) for children <2 years of age (n=5), 31 weeks (range 19 to 63 weeks) for children 2 to <12 years of age (n=10), and 38 weeks (range 1 to 69 weeks) for patients 12 to <18 years of age (n=4). Fifty-three percent of pediatric patients had an identified complement regulatory factor mutation or auto-antibody.

Overall, the efficacy results for these pediatric patients appeared consistent with what was observed in patients enrolled in Studies C08-002A/B and C08-003A/B (Table 16). No pediatric patient required new dialysis during treatment with Soliris.

Table 16: Efficacy Results in Pediatric Patients Enrolled in Study C09-001r

Efficacy Parameter	<2 yrs (N=5)	2 to <12 yrs (N=10)	12 to <18 yrs (N=4)	Total (N=19)
Complete TMA response, n (%)	2 (40)	5 (50)	1 (25)	8 (42)
Patients with eGFR improvement ≥ 15 mL/min/1.73 m^2, n (%)[2]	2 (40)	6 (60)	1 (25)	9 (47)
Platelet count normalization, n (%)[1]	4 (80)	10 (100)	3 (75)	17 (89)
Hematologic Normalization, n (%)	2 (40)	5 (50)	1 (25)	8 (42)
Daily TMA intervention rate, median (range) Before eculizumab On eculizumab treatment	 1 (0, 2) <1 (0, <1)	<1 (0.07, 1.46) 0 (0, <1)	 <1 (0, 1) 0 (0, <1)	 0.31 (0.00, 2.38) 0.00 (0.00 , 0.08)

[1.] Platelet count normalization was defined as a platelet count of at least 150,000 X 10^9/L on at least two consecutive measurements spanning a period of at least 4 weeks.

[2.] Of the 9 patients who experienced an eGFR improvement of at least 15 mL/min/1.73 m^2, one received dialysis throughout the study period and another received Soliris as prophylaxis following renal allograft transplantation.

Adult Patients with aHUS (Study C10-004)

Study C10-004 enrolled patients who displayed signs of thrombotic microangiopathy (TMA). In order to qualify for enrollment, patients were required to have a platelet count < lower limit of normal range (LLN), evidence of hemolysis such as an elevation in serum LDH, and serum creatinine above the upper limits of normal, without the need for chronic dialysis. The median patient age was 35 (range: 18 to 80 years). All patients enrolled in Study C10-004 were required to have ADAMTS13 activity level above 5%;

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observed range of values in the trial were 28%-116%. Fifty-one percent of patients had an identified complement regulatory factor mutation or auto-antibody. A total of 35 patients received PE/PI prior to eculizumab. Table 17 summarizes the key baseline clinical and disease-related characteristics of patients enrolled in Study C10-004.

Table 17: Baseline Characteristics of Patients Enrolled in Study C10-004

Parameter	Study C10-004 (N=41)
Time from aHUS diagnosis until start of study drug in months, median (range)	0.79 (0.03 □311)
Time from current clinical TMA manifestation until first study dose in months, median (range)	0.52 (0.03-19)
Baseline platelet count ($\times 10^9$/L), median (range)	125 (16 □332)
Baseline LDH (U/L), median (range)	375 (131 □3318)

Patients in Study C10-004 received Soliris for a minimum of 26 weeks. In Study C10-004, the median duration of Soliris therapy was approximately 50 weeks (range: 13 weeks to 86 weeks).

Renal function, as measured by eGFR, was improved during Soliris therapy. The mean eGFR (± SD) increased from 17 ± 12 mL/min/1.73m^2 at baseline to 47 ± 24 mL/min/1.73m^2 by 26 weeks. Twenty of the 24 patients who required dialysis at study baseline were able to discontinue dialysis during Soliris treatment.

Reduction in terminal complement activity and an increase in platelet count relative to baseline were observed after commencement of Soliris. Soliris reduced signs of complement-mediated TMA activity, as shown by an increase in mean platelet counts from baseline to 26 weeks. In Study C10-004, mean platelet count (± SD) increased from 119 ± 66 x10^9/L at baseline to 200 ± 84 x10^9/L by one week; this effect was maintained through 26 weeks (mean platelet count (± SD) at week 26: 252 ± 70 x10^9/L). In Study C10-004, responses to Soliris were similar in patients with and without identified mutations in genes encoding complement regulatory factor proteins or auto-antibodies to factor H.

Table 18 summarizes the efficacy results for Study C10-004.

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Efficacy Parameter	Study C10-004 (N=41)
Complete TMA response, n (%), 95% CI Median duration of complete TMA response, weeks (range)	23 (56) 40,72 42 (6, 75)
Patients with eGFR improvement ≥ 15 mL/min/1.73m^2, n (%)	22 (54)
Hematologic Normalization, n (%) Median duration of hematologic normalization, weeks (range)	36 (88) 46 (10, 75)
TMA Event-free Status, n (%)	37 (90)
Daily TMA Intervention Rate, median (range) Before eculizumab On eculizumab treatment	 0.63 (0, 1.38) 0 (0, 0.58)

Pediatric and Adolescent Patients with aHUS (Study C10-003)

Study C10-003 enrolled patients who were required to have a platelet count < lower limit of normal range (LLN), evidence of hemolysis such as an elevation in serum LDH above the upper limits of normal, serum creatinine level ≥97 percentile for age without the need for chronic dialysis. The median patient age was 6.5 (range: 5 months to 17 years). Patients enrolled in Study C10-003 were required to have ADAMTS13 activity level above 5%; observed range of values in the trial were 38%-121%. Fifty percent of patients had an identified complement regulatory factor mutation or auto-antibody. A total of 10 patients received PE/PI prior to eculizumab. Table 19 summarizes the key baseline clinical and disease-related characteristics of patients enrolled in Study C10-003.

Table 19: Baseline Characteristics of Patients Enrolled in Study C10-003

Parameter	Patients 1 month to <12 years (N=18)	All Patients (N=22)
Time from aHUS diagnosis until start of study drug in months, median (range)	0.51 (0.03 □58)	0.56 (0.03-191)
Time from current clinical TMA manifestation until first study dose in months, median (range)	0.23 (0.03 □4)	0.2 (0.03-4)
Baseline platelet count (x 10^9/L), median (range)	110 (19-146)	91 (19-146)
Baseline LDH (U/L) median (range)	1510 (282-7164)	1244 (282-7164)

Patients in Study C10-003 received Soliris for a minimum of 26 weeks. In Study C10-003, the median duration of Soliris therapy was approximately 44 weeks (range: 1 dose to 88 weeks).

Renal function, as measured by eGFR, was improved during Soliris therapy. The mean eGFR (± SD) increased from 33 ± 30 mL/min/1.73m^2 at baseline to 98 ± 44

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mL/min/1.73m^2 by 26 weeks. Among the 20 patients with a CKD stage ≥2 at baseline, 17 (85%) achieved a CKD improvement of ≥1 stage. Among the 16 patients ages 1 month to <12 years with a CKD stage ≥2 at baseline, 14 (88%) achieved a CKD improvement by ≥1 stage. Nine of the 11 patients who required dialysis at study baseline were able to discontinue dialysis during Soliris treatment. Responses were observed across all ages from 5 months to 17 years of age.

Reduction in terminal complement activity was observed in all patients after commencement of Soliris. Soliris reduced signs of complement-mediated TMA activity, as shown by an increase in mean platelet counts from baseline to 26 weeks. The mean platelet count (± SD) increased from 88 ± 42 x10^9/L at baseline to 281 ± 123 x10^9/L by one week; this effect was maintained through 26 weeks (mean platelet count (±SD) at week 26: 293 ± 106 x10^9/L). In Study C10-003, responses to Soliris were similar in patients with and without identified mutations in genes encoding complement regulatory factor proteins or auto-antibodies to factor H.

Table 20 summarizes the efficacy results for Study C10-003.

Table 20: Efficacy Results for Study C10-003

Efficacy Parameter	Patients 1 month to <12 years (N=18)	All Patients (N=22)
Complete TMA response, n (%) 95% CI Median Duration of complete TMA response, weeks (range)[1]	11 (61) 36, 83 40 (14, 77)	14 (64) 41, 83 37 (14, 77)
eGFR improvement ≥15 mL/min/ 1.73•m^2 n (%)	16 (89)	19 (86)
Complete Hematologic Normalization, n (%) Median Duration of complete hematologic normalization, weeks (range)	14 (78) 38 (14, 77)	18 (82) 38 (14, 77)
TMA Event-Free Status, n (%)	17 (94)	21 (95)
Daily TMA Intervention rate, median (range) Before eculizumab treatment On eculizumab treatment	0.2 (0, 1.7) 0 (0, 0.01)	0.4 (0, 1.7) 0 (0, 0.01)

[1] Through data cutoff (October 12, 2012).

14.3 Generalized Myasthenia Gravis (gMG)

The efficacy of Soliris for the treatment of gMG was established in gMG Study 1 (NCT01997229), a 26-week randomized, double-blind, parallel-group, placebo-controlled, multi-center trial that enrolled patients who met the following criteria at screening:

1. Positive serologic test for anti-AChR antibodies,

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2. Myasthenia Gravis Foundation of America (MGFA) Clinical Classification Class II to IV,
3. MG-Activities of Daily Living (MG-ADL) total score ≥6,
4. Failed treatment over 1 year or more with 2 or more immunosuppressive therapies (ISTs) either in combination or as monotherapy, or failed at least 1 IST and required chronic plasmapheresis or plasma exchange (PE) or intravenous immunoglobulin (IVIg).

A total of 62 patients were randomized to receive Soliris treatment and 63 were randomized to receive placebo. Baseline characteristics were similar between treatment groups, including age at diagnosis (38 years in each group), gender [66% female (eculizumab) versus 65% female (placebo)], and duration of gMG [9.9 (eculizumab) versus 9.2 (placebo) years]. Over 95% of patients in each group were receiving acetylcholinesterase (AchE) inhibitors, and 98% were receiving immunosuppressant therapies (ISTs). Approximately 50% of each group had been previously treated with at least 3 ISTs.

Soliris was administered according to the recommended dosage regimen [*see Dosage and Administration (2.4)*].

The primary efficacy endpoint for gMG Study 1 was a comparison of the change from baseline between treatment groups in the Myasthenia Gravis-Specific Activities of Daily Living scale (MG-ADL) total score at Week 26. The MG-ADL is a categorical scale that assesses the impact on daily function of 8 signs or symptoms that are typically affected in gMG. Each item is assessed on a 4-point scale where a score of 0 represents normal function and a score of 3 represents loss of ability to perform that function (total score 0-24). A statistically significant difference favoring Soliris was observed in the mean change from baseline to Week 26 in MG-ADL total scores [-4.2 points in the Soliris-treated group compared with -2.3 points in the placebo-treated group (p=0.006)].

A key secondary endpoint in gMG Study 1 was the change from baseline in the Quantitative Myasthenia Gravis (QMG) total score at Week 26. The QMG is a 13-item categorical scale assessing muscle weakness. Each item is assessed on a 4-point scale where a score of 0 represents no weakness and a score of 3 represents severe weakness (total score 0-39). A statistically significant difference favoring Soliris was observed in the mean change from baseline to Week 26 in QMG total scores [-4.6 points in the Soliris-treated group compared with -1.6 points in the placebo-treated group (p=0.001)].

The results of the analysis of the MG-ADL and QMG from gMG Study 1 are shown in Table 21.

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Table 21: Analysis of Change from Baseline to Week 26 in MG-ADL and QMG Total Scores in gMG Study 1

Efficacy Endpoints	Soliris-LS Mean (N=62) (SEM)	Placebo-LS Mean (N=63) (SEM)	Soliris change relative to placebo □LS Mean Difference (95% CI)	p-values
MG-ADL	-4.2 (0.49)	-2.3 (0.48)	-1.9 (-3.3, -0.6)	(0.006[a]; 0.014[b])
QMG	-4.6 (0.60)	-1.6 (0.59)	-3.0 (-4.6, -1.3)	(0.001[a]; 0.005[b])

SEM= Standard Error of the Mean;

Soliris-LSMean = least square mean for the treatment group;

Placebo-LSMean = least square mean for the placebo group;

LSMean-Difference (95% CI) = Difference in least square mean with 95% confidence interval;

p-values (testing the null hypothesis that there is no difference between the two treatment arms a: in least square means at Week 26 using a repeated measure analysis; b: in ranks at Week 26 using a worst rank analysis).

In gMG Study 1, a clinical response was defined in the MG-ADL total score as at least a 3-point improvement and in QMG total score as at least a 5-point improvement. The proportion of clinical responders at Week 26 with no rescue therapy was statistically significantly higher for Soliris compared to placebo for both measures. For both endpoints, and also at higher response thresholds (≥4-, 5-, 6-, 7-, or 8-point improvement on MG-ADL, and ≥6-, 7-, 8-, 9-, or 10-point improvement on QMG), the proportion of clinical responders was consistently greater for Soliris compared to placebo. Available data suggest that clinical response is usually achieved by 12 weeks of Soliris treatment.

14.4 Neuromyelitis Optica Spectrum Disorder (NMOSD)

The efficacy of Soliris for the treatment of NMOSD was established in NMOSD Study 1 (NCT01892345), a randomized, double-blind, placebo-controlled trial that enrolled 143 patients with NMOSD who were anti-AQP4 antibody positive and met the following criteria at screening:

1. History of at least 2 relapses in last 12 months or 3 relapses in the last 24 months, with at least 1 relapse in the 12 months prior to screening,
2. Expanded Disability Status Scale (EDSS) score ≤ 7 (consistent with the presence of at least limited ambulation with aid),
3. If on immunosuppressive therapy (IST), on a stable dose regimen,

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Long-term treatment with eculizumab in paroxysmal nocturnal hemoglobinuria: sustained efficacy and improved survival

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Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal hematopoietic disorder with increased mortality and morbidity resulting from intravascular hemolysis. Eculizumab, a monoclonal antibody against the complement protein 5, stops the intravascular hemolysis in PNH. We evaluated 79 consecutive patients treated with eculizumab in Leeds between May 2002 and July 2010. The survival of patients treated with eculizumab was not different from age- and sex-matched normal controls ($P = .46$) but was significantly better than 30 similar patients managed before eculizumab ($P = .030$). Three patients on eculizumab, all over 50 years old, died of causes unrelated to PNH. Twenty-one patients (27%) had a thrombosis before starting eculizumab (5.6 events per 100 patient-years) compared with 2 thromboses on eculizumab (0.8 events per 100 patient-years; $P < .001$). Twenty-one patients with no previous thrombosis discontinued warfarin on eculizumab with no thrombotic sequelae. Forty of 61 (66%) patients on eculizumab for more than 12 months achieved transfusion independence. The 12-month mean transfusion requirement reduced from 19.3 units before eculizumab to 5.0 units in the most recent 12 months on eculizumab ($P < .001$). Eculizumab dramatically alters the natural course of PNH, reducing symptoms and disease complications as well as improving survival to a similar level to that of the general population. (*Blood*. 2011;117(25):6786-6792)

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Disclosures

Richard J. Kelly, Anita Hill, Louise M. Arnold, Stephen J. Richards, and Peter Hillmen have received speaker fees from Alexion Pharmaceuticals. Richard J. Kelly, Anita Hill, Stephen J. Richards, and Peter Hillmen have sat on advisory committees for Alexion Pharmaceuticals. Peter Hillmen has received research funding from Alexion Pharmaceuticals. The remaining authors; the Associate Editor Grover C. Bagby; and the CME questions author Laurie Barclay, MD, freelance writer and reviewer, Medscape, LLC, declare no competing financial interests.

Learning objectives

Upon completion of this activity, participants will be able to:

1. Describe survival in patients with PNH treated with eculizumab, according to a case series of 79 consecutive patients treated with eculizumab in Leeds between May 2002 and July 2010.
2. Describe thrombotic episodes in patients with PNH treated with eculizumab according to that case series.
3. Describe transfusion requirements in patients with PNH treated with eculizumab according to that case series.

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Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare, acquired, clonal disorder of hematopoietic stem cells in which the abnormal cells have somatic mutations of the phosphatidylinositol glycan complementation class A (*PIG-A*) gene.[1] Mutations of the *PIG-A* gene disrupt the first step of glycophosphatidylinositol (GPI) biosynthesis, leading to an absence of the GPI anchor and, in turn, a deficiency of all GPI-linked proteins.[2] CD55 and CD59 are both GPI-linked complement regulatory proteins on the surface of blood

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cells, and their absence from erythrocytes renders affected cells susceptible to complement attack causing intravascular hemolysis.[3] Intravascular hemolysis in PNH causes the release of free hemoglobin, leading to the development of anemia, hemoglobinuria, thrombosis, dysphagia, abdominal pain, pulmonary hypertension, renal impairment, and erectile dysfunction.[4-6] Affected persons have chronic hemolysis with exacerbations of the disease where their symptoms become more severe.

PNH can present at any age but mainly occurs in young adults, with the median age at diagnosis being in the early 30s.[7,8] Historical studies have shown the median survival to be between 10 and 15 years from the time of diagnosis.[9,10] More recently, data from a French series of patients have shown an improvement in median survival to 22 years, which is probably the result of improvements in both supportive care and in the management of disease complications.[11] Thrombosis is the main cause of death in PNH. Thrombotic events occur in approximately 50% of patients and result in the deaths of approximately one-third of patients with PNH.[9,10]

Eculizumab is a humanized monoclonal antibody that binds to complement protein 5, thereby inhibiting the formation of the terminal components of the complement cascade.[12] Eculizumab was licensed by the Food and Drug Administration in March 2007 and by the European Medicines Agency in June 2007 for the treatment of PNH after being shown to be well tolerated and highly effective for patients with PNH in clinical trials. It demonstrated a reduction in hemolysis, stabilization of hemoglobin levels, reduction in transfusion requirements, and improvement in quality of life.[13-15] Eculizumab also protects against the complications of hemolytic PNH, such as deteriorating renal function, pulmonary hypertension, and thromboembolism.[16-18]

We report on 79 patients treated with eculizumab at a single center between May 2002 and July 2010 with the first definitive evidence that eculizumab improves survival and effects a sustained improvement in symptoms.

Methods

Patients and investigations

Seventy-nine patients with PNH were treated with eculizumab at Leeds Teaching Hospitals PNH center between May 2002 and July 2010, including 34 patients from the clinical trials[13-15] and a further 45 treated since then. Funding was through the National Health Service either via central commissioning in England or local funding in Wales or Scotland. The follow-up of patients was approved through the Central Research Ethics Committee, and all patients gave signed informed consent for entry into the study in accordance with the Declaration of Helsinki. The diagnosis was established or confirmed using multicolor flow cytometry of the erythrocytes and granulocytes in all cases. Patients were assessed at a minimum of every 12 weeks by the Leeds PNH team. Data collected for analysis include age and symptoms at diagnosis, presence of a history of preceding aplastic anemia (AA) or myelodysplasia (MDS), age at the start of eculizumab, use of other medication to treat PNH, the occurrence of thrombosis before and during eculizumab treatment, blood transfusion requirement for the 12 months before starting eculizumab and after its initiation, lactate dehydrogenase (LDH) levels at the start of eculizumab and the most recent level (all of which were from May, June, or July 2010), and documentation of cause and date of death if applicable. Data were collected up until the end of July 2010 when this planned analysis was made.

In the 7 years before the availability of eculizumab, 30 patients who fulfilled our criteria for eculizumab therapy were cared for in Leeds. The mortality of these patients, up to the point at which they went on to receive eculizumab, was evaluated to provide a control group for comparison.

Eculizumab therapy

Seventy-six patients fulfilled the agreed nationally commissioned indications for treatment: Transfusion-dependent hemolysis (4 or more transfusions in 12 months). A significant PNH-related complication (ie, thrombosis or renal failure) regardless of transfusion history. In addition, 4 patients who did not fulfill these criteria were treated as agreed exceptions requiring therapy because of profound symptoms.

Eculizumab was initially delivered at the approved dosing schedule of 600-mg intravenous infusions more than 30 minutes each week for 4 doses followed by a 900-mg infusion after a further week. A 900-mg dose was then given every 14 days (± 2 days) indefinitely. After the initial 2 to 5 doses, all therapy was administered in the patient's home by homecare nurses (Healthcare-at-Home Limited) under the guidance of the Leeds PNH team. A return of a patient's symptoms (eg, red or black urine or abdominal discomfort) or increased LDH immediately before a dose of eculizumab was indicative of break-through from complement control, and these patients were treated with higher doses (usually 1200 mg) every 14 days.

The major potential complication of eculizumab is the increased risk of meningococcal infection with *Neisseria meningitidis* as terminal complement appears critical to prevent such infections. All patients were vaccinated with a tetravalent meningococcal vaccine against subgroup A, C, W, and Y, but no vaccine is currently available to serogroup B. In the United Kingdom, serotype B is the commonest serotype isolated in meningococcal sepsis. In view of this, in January 2010 our policy changed to continue with vaccination but to also recommend antibiotic prophylaxis to all patients (penicillin V 500 mg twice a day or erythromycin 500 mg twice a day for patients intolerant of penicillin). *N meningitidis* is very sensitive to penicillin; and in areas where there is a high prevalence of serotype B, the use of prophylactic antibiotics may prove to be of benefit.

Statistical analysis

Unless otherwise stated, all analysis was generated using SAS software, Version 9.2. Survival curves were derived by the Kaplan-Meier method using the log-rank test to evaluate the significance of differences between groups. Patient survival on eculizumab was compared with age- and sex-matched control averages obtained using 2001 United Kingdom census data from the United Kingdom Office of National Statistics. To enable a log-rank comparison between these data and the PNH cohort, United Kingdom individual survival data were generated to match the age and sex control averages derived from the census data. A Cox regression model with time-dependent covariates was used to assess whether the introduction of eculizumab as a new treatment strategy altered the mortality of patients with PNH. This analysis included patients with PNH treated in the 7 years before the availability of eculizumab; analysis was generated using Stata Statistical Software release 11. When drawing the survival curve for this pre-ecluzimab cohort, patients were censored at the time they first received eculizumab.

Seventy-five patients required transfusions before eculizumab therapy, and 61 of these (81%) have been on therapy for at least 12 months. Their transfusion requirements in the 12 months before eculizumab and in the most recent 12 months were compared using the Wilcoxon signed rank-sum test. The patients within this group who were not transfusion independent at the time of analysis were further evaluated using a paired *t* test to assess whether there was a significant difference in the number of transfusions required compared with their transfusion requirements before eculizumab.

Thrombotic event rates for pre-eculizumab and eculizumab treatment periods were calculated as the mean number of thrombotic events per year since diagnosis or start of treatment, respectively. Comparison of the occurrence of thrombosis before and after starting eculizumab was carried out using the Wilcoxon signed rank-sum test.

Platelet counts at the start of eculizumab treatment were compared with those after 12 months of treatment using a paired *t* test.

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Table 1. Patient demographics and features at diagnosis and the start of eculizumab treatment (n = 79 unless otherwise stated)

Demographics and features	Value
Presenting features	
Male, no. (%)	40 (51)
Age at diagnosis, y, median (range)	37 (12-79)
Documented history of AA/MDS,* no. (%)	24 (30)
Hemoglobinuria, no. (%)	50 (63)
Abdominal pain, no. (%)	24 (30)
Dysphagia, no. (%)	9 (11)
Thrombosis, no. (%)	4 (5)
Anemia (n = 73),† no. (%)	69 (95)
LDH,‡ IU/L, median (range)	2872 (587-10 300)
Thromboses before eculizumab (n)	
Venous	
Budd-Chiari	12
Mesenteric	4
Cerebral	3
Pulmonary embolus	3
Deep vein	4
Other	3
Arterial	
Stroke	3
Myocardial infarction	2
At start of eculizumab	
Age, y, median (range)	46 (14-84)
LDH level, IU/L, median (range)	2872 (587-10 300)
Neutrophils, $\times 10^9$/L, median (range)	2.2 (0.6-11.9)
Platelets, $\times 10^9$/L, median (range)	149 (11-507)
Reticulocytes (absolute), $\times 10^9$/L, median (range)	171 (57-415)
PNH clone sizes, % (range)	
Granulocyte	96.4 (41.8-100)
Erythrocte	34.0 (2.9-100)
Type II erythrocyte§	3.8 (0-77.4)
Type III erythrocyte§	25.0 (2.4-79.6)
Treatment at start of eculizumab, no. (%)	
Anticoagulation	46 (58)
Ciclosporin	11 (14)
Androgens	2 (3)
No transfusion support‖	4 (5)

*One patient had myelodysplasia.

†Hemoglobin at presentation was not known in 6 cases.

‡Normal value, 430 IU/L.

§Type II erythrocytes have a partial deficiency, and type III erythrocytes have a complete deficiency of GPI-linked proteins.

‖Two patients refused to have blood transfusions for religious reasons.

Results

Patient characteristics

This study includes all patients treated at our center with eculizumab between May 2002 and July 2010. There were 40 men and 39 women with the median age at diagnosis of 37 years (range, 12-79 years) and the median age at initiation of eculizumab of 46 years (range, 14-84 years). Patient characteristics, neutrophil and platelet counts, and PNH clone sizes at the initiation of eculizumab are shown in Table 1. The mean duration of eculizumab treatment was 39 months (range, 1-98 months). Eculizumab has been stopped in 2 patients: one because of predominant aplastic anemia and one with spontaneous remission of his PNH. Seventy-four patients remained on eculizumab until the end of the study date. Seventy-four patients were treated with a maintenance dose of 900 mg of eculizumab every 14 days, with 5 patients requiring higher doses. Four of these required 1200 mg and 1 required 1500 mg every 14 days.

Survival

The survival curve for the patient group compared with age- and sex-matched control averages from the United Kingdom population is shown in Figure 1. There is no significant difference between the 2 groups ($P = .46$). The difference between the curves at 5 years is 0.8% (95% confidence interval [CI], −4.2% to 5.8%). The difference at 8 years is −0.8% (95% CI, −5.8% to 4.2%). Age was significant as a continuous variable in the Cox model for survival ($P = .04$), and none of the 45 patients who commenced eculizumab under the age of 50 years has died. The survival curves for different age groups for PNH patients on eculizumab are shown in Figure 2.

The survival of 30 patients with PNH assessed between 1997 and 2004 who fulfilled the criteria for treatment with eculizumab was also compared with the treated patient group and is shown in Figure 3. The P value obtained from the time-dependent Cox model is .030, with a corresponding hazard ratio of 0.21 (95% CI, 0.05-0.88), suggesting that the risk of dying is significantly reduced on eculizumab. The 5-year survival rate for these patients is 66.8% (95% CI, 41.4%-85.1%), which is significantly worse than the 5-year survival rate for the patients treated with eculizumab, 95.5% (95% CI, 87.6%-98.5%).

Cause of death

Three patients (4%) died while on eculizumab, all from causes unrelated to their PNH. A 55-year-old man died of metastatic cecal carcinoma, which was diagnosed as disseminated disease before eculizumab treatment. A 76-year-old woman died of pneumonia after a long history of recurrent bronchopneumonia before starting eculizumab, and a 79-year-old man with a preceding history of ischemic heart disease died of congestive cardiac failure.

Complications of PNH

Three patients (4%) have undergone a clonal change in their disease. Two patients (age, 71 and 75 years) have developed MDS, 58 and 76 months, respectively, after commencing eculizumab. In both cases, the MDS occurred within the GPI-negative cells; but in each case, the majority of their myeloid series was derived from the PNH clone (granulocyte clone, 100% and 99.4%, respectively). Bone marrow examination in both these patients showed hypercellular marrows with refractory cytopenia and multilineage dysplasia. Cytogenetics in both cases were normal. Another patient (age, 50 years) developed acute myeloid leukemia, 27 years after his initial diagnosis of PNH. The leukemia also developed from GPI-deficient cells; but again, his myeloid series was largely PNH (PNH granulocyte population, 97.9%), and monosomy 7 had been identified in his marrow immediately before commencing eculizumab. The patient with acute myeloid leukemia is undergoing intensive chemotherapy for his leukemia. All 3 patients remained on eculizumab to the end of July 2010.

The relationship between PNH and AA was a prominent feature in this patient cohort, with 11 patients treated with long-term cyclosporine therapy before starting eculizumab and a further 3 patients developing aplasia after eculizumab was initiated. Two of these patients were treated with antilymphocyte globulin and cyclosporine and the third with campath and cyclosporine. All 3 continued eculizumab treatment as they all continued to have

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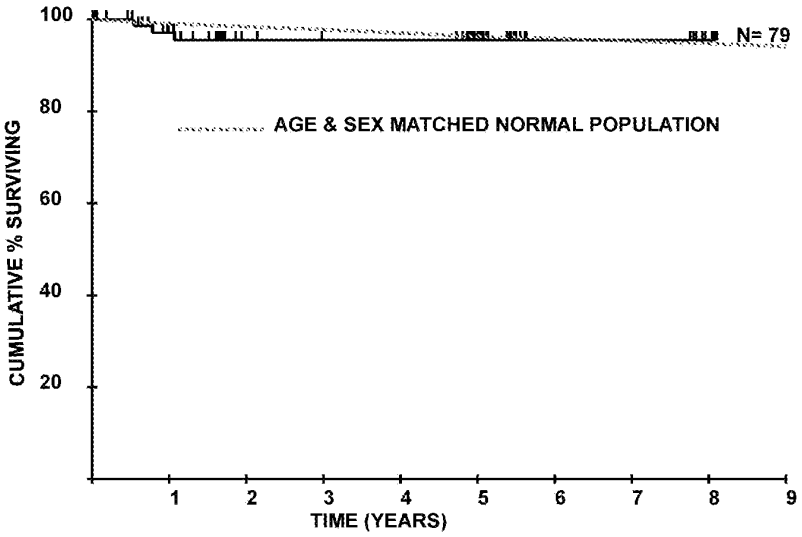


Figure 1. Overall survival of 79 patients from initiation of eculizumab treatment compared with an age- and sex-matched normal population.

significant PNH clones (all 3 had a PNH granulocyte level of > 99.5%).

There were 34 thrombotic episodes in 21 of the 79 patients (27%) before eculizumab treatment. The sites of these thromboses are shown in Table 1. Twenty-three of these were the primary thrombotic events; and in 17 of these cases, the patients were not anticoagulated (74%). Eleven thrombotic events occurred in patients who had already had a previous proven thrombosis, and all of these patients were also on therapeutic anticoagulation. Seven patients had thrombosis within the 12 months before starting eculizumab. Two patients stopped eculizumab temporarily: one because of difficulty in obtaining funding for treatment and the second to establish whether a seronegative arthropathy, which had developed, might improve off treatment (which it did not). Both patients needed to stop their prophylactic anticoagulation because of serious bleeding complications, and both developed thrombotic events within 5 months of stopping eculizumab. These thromboses are not included in the statistical analysis as they occurred in patients no longer on eculizumab.

Two patients had a thrombosis while on eculizumab. The first, who had a stroke before eculizumab, had a retinal vein thrombosis despite treatment with both eculizumab and warfarin. The second, who is on hemodialysis for end-stage renal failure and had no prior history of thrombosis, developed a thrombus in the arteriovenous fistula that had been created.

The number of thromboses occurring from the date of PNH diagnosis to the start of eculizumab treatment was compared with the number occurring while on eculizumab therapy. The mean length of time between PNH diagnosis and starting eculizumab was 7.7 years (range, 0-44 years), and the mean length of time on eculizumab was 3.3 years (range, 0-8 years). The rate of thrombotic events occurring before eculizumab was 5.6 events per 100 patient-years compared with at a rate of 0.8 events per 100 patient-years while on eculizumab ($P < .001$ by the Wilcoxon signed rank-sum test).

Before the advent of eculizumab, primary prophylaxis with warfarin before the patient's first thrombosis was usually recommended in view of the high-risk morbidity and mortality associated with thrombosis in PNH. Because eculizumab appears to be

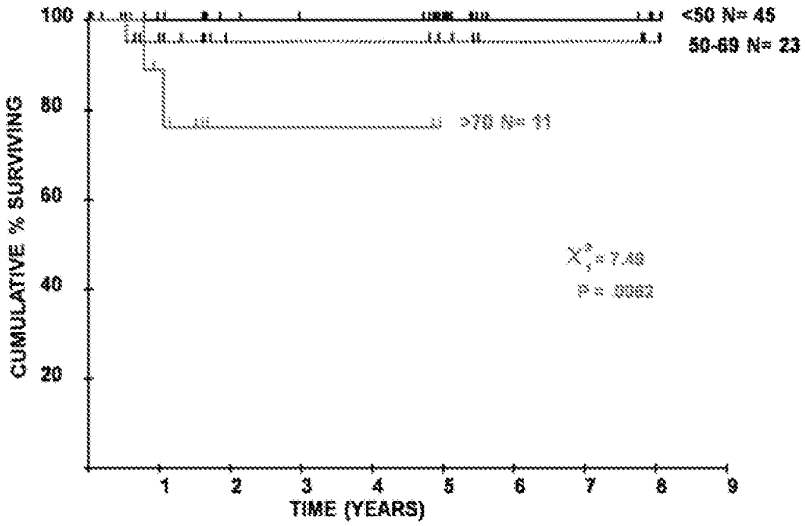


Figure 2. Overall survival of 79 patients from initiation of eculizumab treatment according to age.

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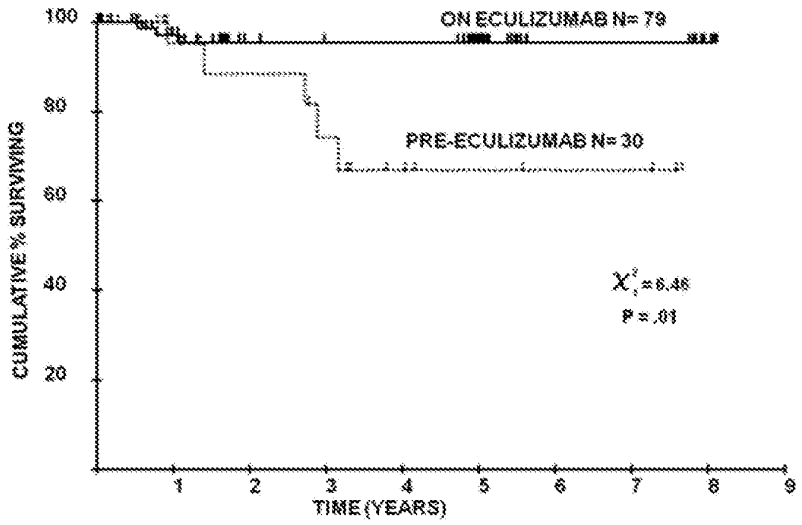


Figure 3. Overall survival of patients before and after eculizumab.

protective against thrombosis in PNH, primary prophylaxis with warfarin has now been stopped in 21 patients and secondary prophylaxis stopped in a further patient because of an episode of life-threatening bleeding. Overall, anticoagulation in patients on eculizumab has been stopped for a mean duration of 10.8 months (range, 1-60 months) and a cumulative total of more than 19 years. No thrombotic complications have been seen in these patients. Anticoagulation of patients with a previous history of thrombosis was not routinely stopped.

Two patients had proven meningococcal infection both with *N meningitidis* serogroup B, which translated to a risk of 0.9 events per 100 patient-years on eculizumab. With advice from the United Kingdom Meningococcal Reference Unit, our policy was altered in January 2010 to include a recommendation for patients to have routine antibiotic prophylaxis.

Intravascular hemolysis and transfusion requirements

The median LDH value fell from 2872 IU/L (range, 587-10 300 IU/L, upper limit of normal 430 IU/L) at the start of eculizumab treatment to the most recent average recording of 477 IU/L (range, 177-1793 IU/L).

In the 12 months before starting eculizumab, the mean number of units of blood transfused was 19.9. Sixty-one of the 75 patients (81%) required transfusions before eculizumab and have been on eculizumab for 12 months. We compared the transfusion requirements for the 12 months before eculizumab therapy with the most recent 12 months on therapy. The mean number of transfusions required fell by 74% from 19.3 units (range, 2-52 units) to 5.0 units (range, 0-50 units, $P < .001$; Figure 4), with 40 of the 61 patients (66%) becoming transfusion independent.

Looking specifically at the 21 patients of this group of 61 who still needed a transfusion in the last 12 months, there was a significant reduction in the number of transfusions needed ($P = .028$). The mean number of units transfused fell from 24.6 units (range, 4-52 units) to 14.6 units (range, 2-50 units). Two of these patients continued to require transfusions as they had developed MDS, 4 patients were transfused as they had breakthrough hemolysis and their eculizumab maintenance dose was increased to 1200 mg every 14 days, 5 patients had 2 or fewer transfusions in the 12-month period (and these were associated with infections usually viral in nature), 2 patients were transfused as they had developed aplasia, and 8 patients continued

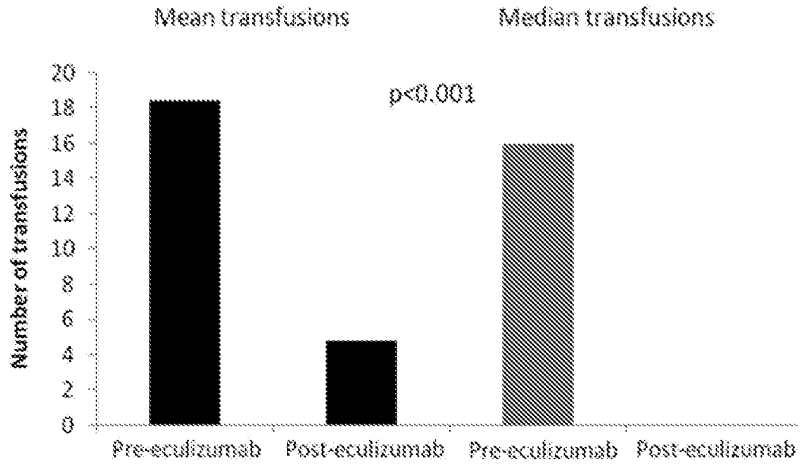


Figure 4. Blood transfusion requirements in the 12 months before eculizumab therapy and the most recent 12 months on eculizumab treatment in 64 patients.

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to need transfusions, which is probably the result of extravascular hemolysis.

Platelets

There are 61 patients who have been on treatment for 12 months and have platelet values recorded both at initiation of eculizumab therapy and after 12 months on eculizumab. There was no difference between platelet counts before or after 12 months of treatment, with the mean platelet count being 161×10^9/L and 163×10^9/L at the start of eculizumab therapy and after 12 months of treatment, respectively. As many patients had increased platelet counts as had decreased platelet counts. We also specifically assessed this in 12 patients with thrombocytopenia (platelets $< 100 \times 10^9$/L) at the start of eculizumab, but there was no difference seen (mean platelet counts, 67×10^9/L and 66×10^9/L at the start of eculizumab therapy and after 12 months of treatment, respectively).

Discussion

This is the first study to show improved survival in patients with PNH treated with eculizumab. We previously published in the era before eculizumab that there was significantly worse survival for patients with PNH compared with matched controls, with approximately 50% of patients dying as a result of their PNH.[9] In the current series, we have shown both a significantly worse survival in patients with PNH in the 7 years immediately before eculizumab ($P = .030$; Figure 3) and no difference in mortality between patients on eculizumab and the normal population ($P = .46$; Figure 1). Three of the 79 patients (4%) treated with eculizumab at our center during the past 8 years have died, all from non–PNH-related causes. There were no deaths in the 45 patients commencing eculizumab under the age of 50 years, which is a significant finding as the median age at diagnosis in PNH is in the fourth decade of life.[7,8] Two of the 11 patients more than 70 years old died as a result of comorbidities and not as a direct result of their PNH (Figure 2).

In our comparison of patients with PNH treated in the 7 years immediately before eculizumab with those patients on eculizumab, the patients effectively act as their own controls, with a period before the introduction of eculizumab and a period after the introduction of eculizumab, which is considered to be an effective analysis strategy. Selection bias is minimized because the criteria for inclusion in these groups are the same (those fulfilling the criteria for eculizumab treatment in England), all presenting patients are included, and all patients were cared for at a single center and were treated with eculizumab as soon as it was available. Although other treatments, such as supportive care and treatment of disease complications, may have improved over that period of time, it is doubtful that these improvements could have affected survival to the extent displayed (Figure 3).

Three patients (4%) developed MDS or acute leukemia, which is similar to the level reported previously.[8,10,11] Two of these patients developed MDS (age, 71 and 75 years, respectively). In both instances, the MDS occurred within the GPI-negative clone of cells. A further patient (age, 50 years) developed acute myeloid leukemia 27 years after his initial diagnosis of PNH. The leukemic blast cells were also GPI-deficient. The fact that clonal myeloid evolution occurred from the PNH clone in these patients is not surprising as the majority of hematopoiesis in these 3 patients was derived from PNH stem cells.

The most common feature at diagnosis in PNH patients is that of anemia,[19] and this is confirmed in our patient group. Hemoglobinuria, the classic symptom seen in PNH, was present in a much higher proportion of our patients at diagnosis (63%) than in previously reported series[20] but is not seen in all PNH patients and can be a contributory factor in the often lengthy delay in a diagnosis being made.[9]

Thromboembolism is the main cause of mortality in PNH, accounting for approximately 40% of deaths.[5,7,9-11] Once an initial thrombosis occurs, the risk of further thrombotic events occurring is much higher, with a 5- to 10-fold increase in mortality.[8,10] Primary prophylaxis with anticoagulation is usually successful[21]; but in cases where an initial thrombosis has occurred, anticoagulation alone is frequently not sufficient to prevent further thrombotic complications.[7,22] In addition, there are well-recognized risks of anticoagulation, particularly in a population of patients with a high incidence of thrombocytopenia and of liver abnormalities.

We observed 34 thrombotic events in 21 patients (27%) before starting eculizumab. Seventeen (50%) of these occurred while on anticoagulation, with 11 of these 17 events occurring in patients with a previous proven thrombotic event. This supports the finding that anticoagulation alone will not always prevent thromboses, especially in those with a previous history of thrombosis. We observed a dramatic reduction in thromboses occurring on eculizumab therapy with just 2 events reported. Rates of thromboses reduced from 5.6 events to 0.8 events per 100 patient-years with eculizumab treatment. Even after accounting for the shorter duration of follow-up since starting eculizumab, this difference is highly significant ($P < .001$).

Seven patients had a thrombosis in the 12 months leading up to the start of eculizumab but have had no further thrombotic events since commencing therapy. It appears that, in this setting, eculizumab in conjunction with anticoagulation is effective in preventing further thrombosis. We have also stopped primary prophylactic anticoagulation in 21 patients on eculizumab with no instances of thrombosis occurring (cumulative time period of > 19 years). We now routinely recommend the cessation of anticoagulation in patients on eculizumab with no prior history of thrombosis. This is important as there is a small but significant risk of severe bleeding on long-term anticoagulation.[23] Anticoagulation of patients with a previous history of thrombosis has not been systematically stopped.

Two patients with large PNH clones stopped eculizumab temporarily. In both cases, anticoagulation was stopped during this time because of specific bleeding complications. Within a few months, both patients had developed a thrombosis and were restarted on eculizumab. Although this has been seen in only 2 cases, if patients have to stop eculizumab but still have a significant disease burden, anticoagulation should be considered before it is stopped. Consideration should also be given to transfusions in patients stopping eculizumab to dilute the PNH red cell population, thereby decreasing the likelihood of hemolysis.

Eculizumab therapy in PNH causes a rapid reduction in intravascular hemolysis with a corresponding reduction in LDH levels,[13-15] which we now demonstrate continues to remain low beyond 8 years of therapy. Two-thirds of patients on eculizumab for at least 12 months remain transfusion independent. This is higher than the proportion of patients who remained transfusion independent in the eculizumab clinical trials, with 51% of patients being transfusion independent for both the 6-month period of the TRIUMPH study and the 12 months of the SHEPHERD study.[14,15] This may reflect an improvement in patients who are treated with eculizumab for a prolonged period, particularly in those patients

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who are acutely unwell when commencing therapy, and/or the patients in our study are more homogeneous because of the strict criteria for eculizumab therapy. In addition, the 21 patients who still require transfusions had a significant reduction in the amount of blood required after starting eculizumab ($P = .03$). This improvement is seen even though 2 of these patients had a marked increase in the amount of blood they required as a result of developing MDS. Patients in our study who still needed transfusions on eculizumab did so for a variety of reasons. These include breakthrough hemolysis, aplasia, and extravascular hemolysis. Extravascular hemolysis has been reported in 2 studies on patients with PNH receiving eculizumab.[24,25] In our series, we observed a dramatic reduction in transfusion requirements, indicating that the phenomenon of extravascular hemolysis is of limited clinical importance for most patients.

An increase in platelet count in patients with thrombocytopenia as a direct consequence of eculizumab treatment has previously been observed.[26] No increase in platelet count was observed in our series, either looking at all study patients or just those with thrombocytopenia.

This study underlines the pivotal role that intravascular hemolysis plays in PNH. Inhibition of the formation of the terminal complement cascade, preventing intravascular hemolysis of PNH erythrocytes and other sequelae of complement on PNH cells, not only alleviates the symptoms of the disease but also improves survival to such an extent that in our patient group there were no deaths because of the sequelae of hemolytic PNH (ie, thrombosis or renal failure). Eculizumab appears to have no effect on the development of clonal myelopathies, namely, MDS or acute myeloid leukemia, which are rare occurrences in PNH; but given this caveat, the observed survival for patients on eculizumab appears to be similar to that of the normal population.

Authorship

Contribution: R.J.K. designed the study, collected, analyzed, and interpreted the data and drafted the paper; L.M.A., G.L.B., S.J.R., M.C., and L.D.M. analyzed and interpreted the data and reviewed the paper; D.R.C. and W.M.G. analyzed, interpreted, and performed statistical analysis on the data and reviewed the paper; and A.H. and P.H. designed the study, analyzed and interpreted the data, and reviewed the paper.

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Minireview

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Antibody Engineering for the Development of Therapeutic Antibodies

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Therapeutic antibodies represent one of the fastest growing areas of the pharmaceutical industry. There are currently 19 monoclonal antibodies in the market that have been approved by the FDA and over 150 in clinical developments. Driven by innovation and technological developments, therapeutic antibodies are the second largest biopharmaceutical product category after vaccines. Antibodies have been engineered by a variety of methods to suit a particular therapeutic use. This review describes the structural and functional characteristics of antibody and the antibody engineering for the generation and optimization of therapeutic antibodies.

Keywords: Affinity Maturation; Antibody Engineering; Antibody Fragments; Effector Functions; Human Monoclonal Antibodies; Humanized Antibodies; Pharmacokinetics; Therapeutic Antibodies.

Introduction

Today, therapeutic monoclonal antibodies (MAbs) represent one of the fastest growing area of the pharmaceutical industry, with exceptional 48.1% growth between 2003 and 2004. There are currently 19 therapeutic antibodies in clinical use that have been approved by the FDA (Fig. 1; Table 1) and over 150 antibodies in clinical trials. The MAbs market is expected to almost triple in value over the next six years from $10.3 billion in 2004 to $30.3 billion in 2010, driven by technological evolution from chimeric and humanized to fully human antibodies. Oncology products will continue to dominate the market. However, sales of arthritis, immune and inflammatory disorders products are forecast to grow strongly and account for 40.1% of the market by 2010. The development focus of the industry is moving away from murine and chimeric antibodies, to humanized and, in particular, fully human antibodies. A wave of fully human antibodies are expected to launch from 2007 onwards.

The features that make antibodies attractive drug candidates are high target specificity and their organization into distinct structural and functional domains. The characteristic domain structure of antibody has facilitated protein engineering for the development of therapeutic antibodies. When an antibody is designed as a drug, all of its different features including immunogenicity, affinity, stability, effector functions, half-life, and tissue penetration and distribution should be taken into consideration and optimized accordingly. From a manufacturing standpoint, ease of production and stability must also be considered. Nowadays, natural antibodies are tailored by a variety of methods to suit a particular therapeutic use. This review describes the structure and function of antibody, and antibody engineering for the generation of humanized antibodies, antibody fragments, and fully human MAbs. In addition, the technologies for enhancing their biological activities such as affinity maturation, improvement of effector functions, and altering pharmacokinetics are reviewed.

Structural and functional features of antibodies

Immunglobulins (Ig) are highly specific and naturally

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Abbreviations: AAR, anti-antibody response; ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; CDR, complementarity determining region; Fab, antigen-binding fragment; FACS, fluorescence-activated cell sorting; Ig, immunoglobulin; MAb, monoclonal antibody; scFv, single-chain Fv; V, variable; VH, heavy chain variable domain; VL, light chain variable domain.

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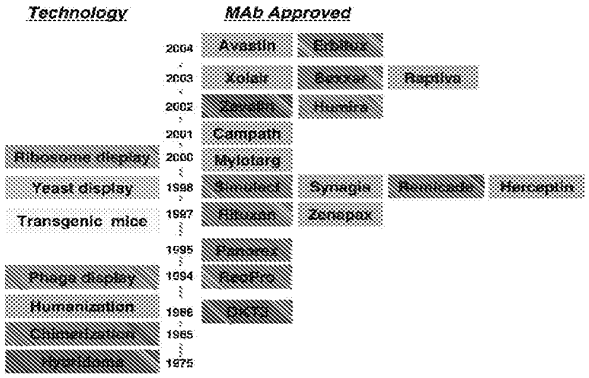


Fig. 1. Antibody technologies and approved therapeutic antibodies.

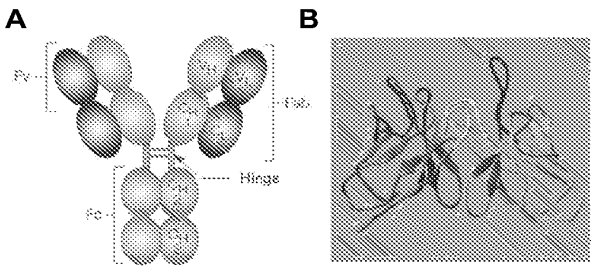


Fig. 2. The modular structure of antibody molecule (A) and the CDR1, 2, and 3 within the heavy and light chain variable domains (B).

evolved molecules that recognize and eliminate foreign antigens. There are five classes of immunoglobulins: IgM, IgG, IgE, IgA, and IgD. From a biotechnology perspective, IgG is the most important class of antibodies. IgG is organized into distinct structural and functional domains (Fig. 2). IgG consists of two identical heavy chains (50 kDa) and two identical light chains (25 kDa). Light chains consist of one V domain (VL) and a single constant domain (CL), whereas heavy chains comprise one V domain (VH) and three constant domains (CH1, CH2, and CH3).

Functionally, antibody is divided into two antigen-binding fragments (Fabs) and a constant (Fc) region, which are linked via a flexible hinge region. The heavy (VH) and light (VL) chain variable domains in the amino-terminal part of Fabs bind antigens. Each domain features the characteristic immunoglobulin fold consisting of two antiparallel β-sheets with an intramolecular disulfide bond. Within each V domain are three regions that are hypervariable in sequence and that form loops at the ends of rigid β-sheets. The hypervariable loops are primarily responsible for antigen recognition and are referred to as complementarity determining regions (CDRs). The remaining V region amino acids act as a scaffold to support the loops and are referred to as framework residues (FR).

The Fc portion of antibody mediates effector functions, antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). In ADCC, antibodies bind to Fc receptors (FcγRs) on the surface of effector cells such as natural killer (NK) cells and macrophages, and trigger phagocytosis or lysis of the targeted cells. In CDC, antibodies kill the targeted cells by triggering the complement cascade at the cell surface. The human IgG1 is most efficient in both CDC and ADCC, and therefore the most suitable for therapeutic use against pathogens or tumor cells. On the other hand, human IgG4 is not active in CDC or ADCC, and therefore may be suitable for diagnostic imaging or blocking molecular interactions. The interaction of IgG with the FcγRs or the first component (C1q) of complement depends on the hinge region and the CH2 domain.

Fc region is associated with serum half-life of antibody. The half-life of human IgG1 is generally 3 weeks, whereas that of antibody fragment is just a few hours. This is because FcRn receptor on the endothelial cells binds the interface between the CH2 and CH3 domains and protects IgG1 from degradation. The most notable feature of this binding is that it is pH-dependent with good binding at slightly acidic pH such as 6.0 which exists in endosomes where the FcRn protects the pinocytosed and endocytosed IgG by binding from degradation. On recycling, the IgG is released into the extracellular regions because there is almost no binding at pH 7.4 of the extracellular space. Therefore, to reduce the half-life of an antibody drug, for example, for radio-imaging, antigen binding fragments instead of whole IgGs can be used.

Humanization of murine antibodies

Since the hybridoma technology for the production of murine MAb was established in 1975, an anti-CD3 murine MAb (OKT3) was approved as the first therapeutic antibody in 1986. Despite the high expectations of MAb therapy, OKT3 failed as a good treatment for transplantation rejection primarily because OKT3 induced severe human anti-murine antibody (HAMA) response in patients.

To reduce the immunogenicity of murine antibodies in humans, chimeric antibodies with mouse variable regions and human constant regions were constructed by genetic engineering (Morrison *et al.*, 1984) (Fig. 3). In 1994, ReoPro, a chimeric Fab, was introduced as the second therapeutic antibody. Since then, five more chimeric antibodies have been marketed and clinically used (Table 1). However, although chimeric antibodies were less immunogenic than murine MAbs, human anti-chimeric antibody (HACAs) responses have been observed (Bell and Kamm, 2000).

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Table 1. Approved therapeutic antibodies.

Year (FDA)	Trade name (Generic)	Type of antibody (Target antigen)	Disease indication	Company
1986	OKT 3 (muromanab-CD3)	Murine (CD3)	Allograft rejection	Ortho Biotech
1994	ReoPro (abciximab)	Chimeric Fab (GPIIb/IIIa)	Adjunct to PTCA	Centocor
1995	Panorex (edrecolomab)	Chimeric (CA17-1A)	Colorectal cancer	GSK/Centocor
1997	Rituxan (rituximab)	Chimeric (CD20)	Non-Hodgkins lymphoma	IDEC
1997	Zenapax (daclizumab)	Humanized (IL2R)	Prevention of kidney transplant rejection	PDL
1998	Herceptin (trastuzumab)	Humanized (Her2/neu)	Metastatic breast cancer	Genentech
1998	Synagis (palivizumab)	Humanized (RSV F)	RSV propylaxis	MedImmune
1998	Simulect (basiliximab)	Chimeric (IL2R)	Prevention of kidney transplant rejection	Norvatis
1998	Remicade (infliximab)	Chimeric (TNF-α)	Rheumatoid arthritis, Crohn's disease	Centocor
2000	Mylotarg (gemtuzumab ozogamicin)	Humanized-calicheamicin(CD33)	CD33-acute myeloid leukemia	Celltech
2001	Campath (alemtuzumab)	Humanized (CD52)	B-cell Chronic Lymphocytic Leukemia	Millennium
2002	Zevalin (ibritumomab tiuxetan)	Murine-Y-90 (CD20)	Non-Hodgkins lymphoma	IDEC
2002	Humira (adalimumab)	Human (TNF-α)	Crohn's disease, RA	CAT/BASF
2003	Xolair(omalizumab)	Humanized (IgE)	Asthma	Tanox/Genentech/ Novartis
2003	Raptiva (efalizumab)	Humanized (CD11a)	Psorisis	Xoma/Genentech
2003	Bexxar (tositumomab)	Murine-I-131 (CD20)	Non-Hodgkins lymphoma	Corixa/GSK
2004	Erbitux (cetuximab)	Chimeric (EGFR)	Colorectal cancer	Imclone
2004	Avastin (bevacizumab)	Humanized (VEGF)	CRC, breast, renal, NSCL cancer	Genentech

To further minimize the mouse component of antibodies, humanized antibodies were constructed by protein engineering (Jones *et al.*, 1986) (Fig. 3). In such 'humanized' antibodies, only the CDR loops that are responsible for antigen binding are grafted onto human variable-domain framework, which is referred to as CDR-grafting. However, simple CDR-grafting often results in significant loss of antigen-binding affinity. Therefore, to restore the affinity of parental murine MAb, key framework residues that support the conformations of CDR loops in the murine antibody are also grafted onto the human template, which is the most important part in the development of humanized antibody by CDR-grafting. Since the first humanized antibody, Zenapax was marketed in 1997, seven more humanized antibodies have been approved as therapeutics and are in clinical use (Table 1).

CDR-grafting has been designed with the aid of computer-guided modeling of antibody variable domain (Tsurushita *et al.*, 2005). Recently, several other approaches to humanization are executed. Hwang *et al.* (2005) select human templates on the basis of structural similarity between human and murine CDRs and identify human templates that can support the grafted murine CDRs in binding to their cognate antigen. Dall'Acqua *et al.* (2005) use framework shuffling in which the CDRs of a murine antibody are fused in frame to pools of synthetic human germline frameworks, and screen the resulting combinatorial library for the combinations most able to support binding. Kashmiri *et al.* (2005) identify the CDR residues most crucial to the antigen-antibody interaction using structural and experimental information and graft those residues, not whole CDRs, onto a human template.

Generation of fully human monoclonal antibodies

While the production of murine MAbs is routine, the production of human MAbs by conventional hybridoma technology has been difficult because human hybridomas and immortalized cell lines do not stably produce high levels of antibody and *in vivo* immunization of humans is

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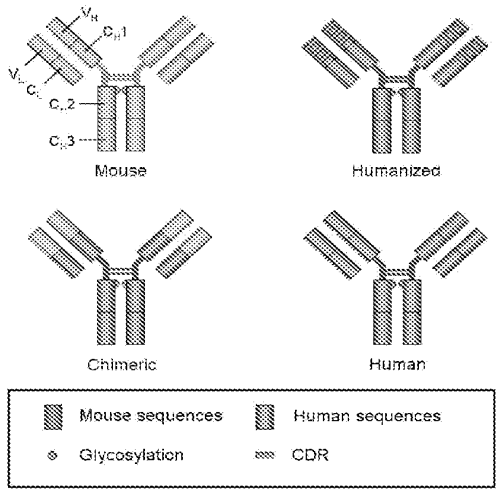


Fig. 3. Antibody engineering for humanization.

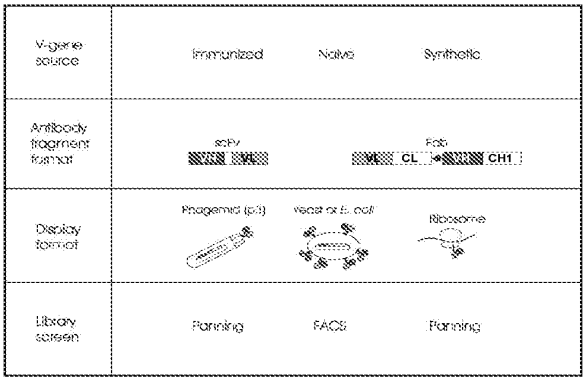


Fig. 4. The display and screening system of antibody libraries.

not feasible for many antigens. However, the development of methods for the expression of antibody fragments such as Fab or ScFv in bacteria and the display of antibody fragments on filamentous bacteriophage as well as powerful techniques for the screening of antibody libraries made it possible to generate human MAbs. This phage-display technology is the most used and well-established technology for the development of new human antibodies (Fig. 4). As an alternative strategy for producing human MAbs, transgenic mice containing human immunoglobulin germ line locus are used. Immunization of such transgenic mice results in a human antibody response, from which hybridomas that produce human antibodies can be generated, as in traditional hybridoma technology.

In 2002, the first human MAb, Humira, developed by phage display, was approved by the FDA and marketed (Table 1). The human MAbs derived from transgenic mice are in clinical trials. Recently, human MAbs are more developed than chimeric or humanized antibodies.

Phage display technology

In the mammalian immune system, antibodies with high affinities are created by combinatorial recombination of germline antibody gene segments and somatic hypermutation in antibody genes, preferentially within the CDRs (Neuberger *et al.*, 1998; Wabl *et al.*, 1999). B-lymphocytes expressing affinity-matured antibodies are then selected and expanded (Kuby, 1997). Phage antibody technology can successfully mimic the immune system by cloning large libraries of antibody genes and selecting for binding to a desired target.

Immune library Humans exposed to a desired antigen through vaccination or disease have high levels of circulating antibodies to the antigen. Therefore, even small libraries ($\sim 10^5$) from immunized donors give rise to specific antibodies. In addition, because the antibody genes have experienced affinity maturation *in vivo*, antibodies that do not require further affinity maturation can potentially be isolated. However, isolation of human MAbs from immune library has certain limitations because human antibodies in general cannot be generated by immunization for ethical reasons. Also, immunological tolerance mechanisms make it very difficult and often impossible to isolate antibodies against self antigens, many of which are potentially important therapeutic targets, particularly in cancer.

Naïve library Naïve libraries represent the V-gene repertoire created by cloning the antibody genes found in non-immunized individuals. mRNA is isolated from peripheral lymphocytes, bone marrow, tonsils, and cadaver spleens. Subsequently, IgM and/or IgG variable regions are amplified from mRNA by PCR using degenerate oligonucleotide primer sets and cloned into vectors suitable for screening. IgM repertoires are preferred to IgG because they have not been subjected to tolerance or antigen selection and are thus more diverse. In contrast, IgG chains can be biased by host immune responses and will not react to self antigens (Griffiths and Duncan, 1998). The affinity and specificity of antibodies that can be isolated from naïve libraries is intrinsically linked to library size. For example, a library consisting of 10^{10} different clones yielded antibodies with affinities in the low nanomolar range (Vaughan *et al.*, 1996), whereas a similar library of 3×10^7 clones only resulted in antibodies with micromolar affinities (Marks *et al.*, 1991). Once a library has been made, it can be propagated and used repeatedly to isolate antibodies against numerous antigens. However, poor expression and toxicity to the host bacteria are often issues with antibodies isolated from naïve libraries. These problems may be circumvented by using

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synthetic antibody repertoire libraries.

Synthetic library In principle, synthetic libraries have potentials of encoding antibodies to self-antigens. To create fully synthetic repertoires, germline antibody gene segments, V_H, D_H, and J_H or $V_{\kappa/\lambda}$ and $J_{\kappa/\lambda}$ are cloned and arranged combinatorially *in vitro* so as to reconstitute genes encoding complete V_H and V_L chains (Winter, 1998). Such synthetic libraries of 10^7–10^{10} clones gave rise to antibodies with specificity to self-antigens (de Kruif *et al.*, 1995; Griffiths *et al.*, 1994; Hoogenboom and Winter, 1992; Nissim *et al.*, 1994), but their affinities are generally not high (Griffiths and Duncan, 1998).

Semi-synthetic libraries have also been generated by selecting one or more antibody frameworks as a scaffold and randomizing sequences within the CDR loops. Because CDR3 contributes most of the antigen-binding contacts, it has been predominantly used as a target for randomization (Barbas *et al.*, 1992). Early semi-synthetic libraries gave rise to antibodies with rather low K_D values for antigen binding, typically in the 100 nM range (de Wildt *et al.*, 1996). Randomization of key residues in the light chain CDRs, in addition to the heavy chain CDR3, has been employed to improve diversity, resulting in the recovery of higher affinity antibodies (Huls *et al.*, 1999).

Recently, Knappik *et al.* (2000) developed fully synthetic Human Combinatorial Antibody Library (HuCAL). They constructed 49 combinations of seven VH and seven VL consensus frameworks that represented each of the human V gene families and were optimized for expression in *E. coli*, then randomized the heavy and light chain CDR3 residues of the 49 master genes to build the synthetic library. This highly diverse library has been used to isolate numerous antibodies with high affinities and good expression characteristics in *E. coli*.

Antibody library screening The screening of combinatorial antibody libraries is one of the most important tools in antibody engineering. Efficient high throughput screening of large libraries has enabled the isolation of specific antibody clones and engineering of antibodies with high affinity, increased stability and improved effector functions. Currently, the most widely used technique for library screening is based on the display of antibodies on the surface of filamentous bacteriophages (Hoogenboom *et al.*, 1998). Antibody library in the Fab or ScFv format is fused to a surface protein of phages, most commonly pIII encoded by the gene III. Phages displaying an antibody specific for an antigen can be readily enriched by selective adsorption onto immobilized antigen, a process known as panning. Then the bound phage is eluted from the surface and amplified through infection of *E. coli* cells. Usually, 5–8 rounds of panning, elution, and amplification are sufficient to select for phages displaying specific antibodies, even from very large libraries (up to 10^{11} clones). A variety of techniques for the efficient enrichment of specific and high-affinity clones have been developed (Duenas and Borrebaeck, 1994; Griffiths and Duncan, 1998; Hawkins *et al.*, 1992; Hoogenboom *et al.*, 1998; Spada *et al.*, 1997).

Cell display Antibodies can also be displayed on the surface of microbial cells such as *E. coli* and *Saccharomyces cerevisiae* (Boder and Wittrup, 1997; Francisco *et al.*, 1993; Fuchs *et al.*, 1991; Georgiou *et al.*, 1997; Gunneriusson *et al.*, 1996). For screening purposes, a library of cells, each displaying multiple copies of a different antibody variant, is incubated with a fluorescently tagged ligand in buffer. Cells displaying antibodies that bind the ligand become fluorescently labeled and are isolated by fluorescence-activated cell sorting (FACS). With flow cytometry, the binding of each clone in the library to a particular ligand is determined quantitatively. Parameters such as ligand concentration or time for the dissociation of antibody:ligand complexes can be easily optimized to ensure the isolation of only the highest affinity antibodies. At present, cell surface display and FACS cannot be used for the de novo isolation of antibodies from natural or synthetic repertoire libraries because the maximum library diversity that can be realistically screened by FACS is about 5×10^8. These features are particularly significant for antibody affinity maturation. However, repertoire libraries can be screened by 1–2 rounds of panning to reduce the library diversity, followed by cell display technologies that guarantee the isolation of high-affinity, well expressed antibodies.

Ribosome display Ribosome display relies on the formation of a ternary complex between ribosomes, mRNA, and the polypeptide (Hanes and Pluckthun, 1999; Hanes *et al.*, 1998; He and Taussig, 1997; Mattheakis *et al.*, 1994). Complexes containing folded antibodies with a desired specificity are enriched by panning against immobilized ligand. The mRNA of the ribosome-mRNA-protein complexes is reverse transcribed to produce the DNA encoding the antibodies responsible for the binding of the complexes to the immobilized ligand. The DNA is then transcribed by RNA polymerase to begin another cycle of ternary complex formation and selection. Alternatively, a covalent link may be established directly between mRNA and the protein it encodes (Roberts and Szostak, 1997). Covalent mRNA-protein complexes are formed via the reactive amino acid analogue puromycin. The advantages of this technology are that covalent mRNA-protein is more stable and can be subjected to harsh screening conditions to enrich antibodies or other proteins with increased stability and the entire process is performed entirely *in vitro* without transformation. Using this technology, Hanes *et al.* (2000) obtained picomolar affinity antibodies from the synthetic HuCAL library.

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Human antibodies from transgenic mice

A radically different approach to antibody humanization is to develop murine hybridomas that produce fully human antibodies. For this purpose, transgenic mice in which the native immunoglobulin repertoire has been replaced with human V-genes in the murine chromosome have been generated (Bruggemann and Taussig, 1997; Fishwild *et al.*, 1996; Mendez *et al.*, 1997). These mice appear to carry out VDJ recombination and somatic hypermutation of the human germline antibody genes in a normal fashion, thus producing high-affinity antibodies with completely human sequences and differing only in glycosylation patterns. The transgenic mice can be injected with a desired antigen (including human proteins), and the resulting antibody genes are recovered by cloning and screening an immune library, or by conventional hybridoma technology. Antibodies with mid-picomolar affinities have been isolated and these appear promising for therapeutic applications (Fishwild *et al.*, 1996; Yang *et al.*, 1999a; 1999b). Importantly, their half-life in *Cynomologus* monkeys was found to be similar to antibodies recovered from humans [9.5–11 days for transgenic mouse-derived human antibodies, versus 9.6 days for human IgG, 1.9 days for murine IgG, and 7.1 days for chimeric mouse-human IgG (Yang *et al.*, 1999a; 1999b)]. These mice present a major advance for hybridoma technology.

Immunogenicity of chimeric, humanized, and fully human antibodies in humans

Recently, Hwang and Foote reviewed reported immunogenicity of murine, chimeric, and humanized antibodies in humans (Hwang and Foote, 2005). They grouped the anti-antibody response (AAR) incidence into three operational categories: *negligible*, *tolerable*, and *marked*. *Negligible* AAR was classified when AAR was reported in less than 2% of patients, which represents an ideal in terms of safety. *Tolerable* AAR was classified when it was detectable in 2–15% of patients. In this case, the MAbs were essentially flawed, though use was arguably warranted for catastrophic or life-limiting disease. *Marked* AAR was classified when AAR was detected in more than 15% of patients. Products with *marked* AAR were usually clinical failures and regulatory concerns were likely to preclude clinical use except for one time use as radioimmunoconjugates. As shown in Fig. 5, among the 44 murine MAbs analyzed, 84%, 7%, or 9% had marked, tolerable, or negligible HAMA responses, respectively. Among the 15 chimeric antibodies with clearly reported AAR, 40%, 27%, or 33% had marked, tolerable, or negligible HACA responses, respectively. On analyzing 22 humanized antibodies with AAR, 9%, 36%, or 55% had marked, tolerable, or negligible HAHA responses, respectively. The

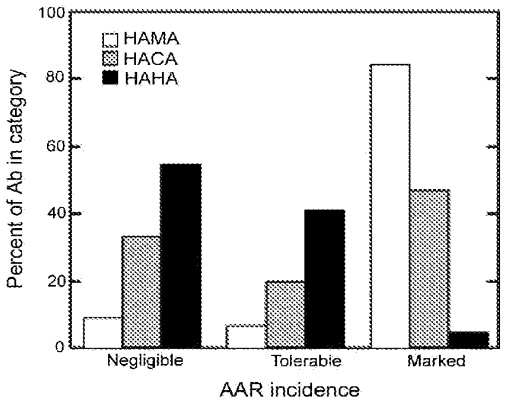


Fig. 5. Anti-antibody response incidence compared to molecular format.

data clearly show that chimerization largely decreases the immunogenicity of therapeutic antibodies and humanization decreases it further. However, both humanized and chimeric antibodies have about equal proportions of tolerable and negligible examples, which suggests that it may be necessary to further reduce the immunogenicity of humanized antibodies. Likewise, whether fully human MAbs are less immunogenic than humanized antibodies or not cannot be answered at present because full immunogenicity data are available for just one MAb Humira, developed from phage-displayed human libraries. Repetitively dosed Humira showed AAR in 12% of patients - at the higher end of HAHA responses. More data on the immunogenicity of human antibodies will be needed to answer the question.

Antibody fragments

Whole antibody with a molecular weight of about 150 kDa diffuses poorly from the vascular bed into a solid tumor mass and clears slowly from the body. Antibody fragments such as Fab, scFv, diabodies, and minibodies can be generated by removing the entire constant region or part or whole of the Fc portion (Fig. 6). These antibody fragments are known to have better clearance from whole body and also better tissue/tumor penetration characteristics. Thus antibody fragments are better suited for imaging and/or radio-therapy (Chang *et al.*, 2002; Colcher *et al.*, 1998; Wu, 2004; Wu and Yazaki, 2000). Antibody fragments are thought to be easy to produce in bacteria in large amounts and are therefore considered to bypass the hurdles associated with mammalian cell based production of whole antibodies.

In general, the smallest fragment of an antibody that retains the antigen binding specificity of whole antibody is the Fv in which the VH and VL domains are noncova-

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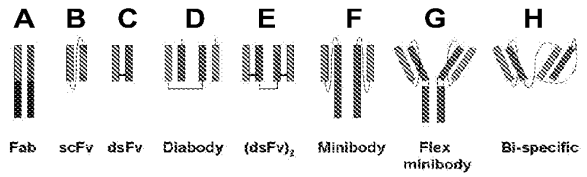


Fig. 6. Schematic representation of antibody fragments.

lently associated, although even single V domain can bind to antigens (Ward *et al.*, 1989). Because of its instability at low concentrations, the VH and VL domains of Fv are linked by a flexible peptide linker to make a single-chain Fv (scFv) (Fig. 5B) (Bird *et al.*, 1988). The most common peptide linker is a flexible $(Gly_4Ser)_3$. Also, Fv is engineered to form a disulfide bond by introducing two cysteine residues in the framework regions of VH and VL to yield a disulfide stabilized Fv (dsFv) (Fig. 5C) (Brinkmann *et al.*, 1993). Converting a whole IgG to a scFv or dsFv is usually associated with a decrease in the antigen binding activity because of the loss of avidity. However, this loss in binding activity can be compensated by engineering the Fvs to increase affinity.

Diabodies are homodimers of scFvs that are covalently linked by a short peptide linker of four amino acids which forces the V domains to make inter-molecular complexes with their cognate domains (Fig. 5D) (Holliger *et al.*, 1993). In a slightly different format called $(dsFv)_2$, the VH and VL are engineered to form a dsFv but the VH is expressed twice in tandem separated by a flexible linker. When the VH and VL are co-expressed, a divalent molecule is formed (Fig. 5E) (Bera *et al.*, 1998). Minibodies are homodimers of scFv-CH3 fusion proteins (Fig. 5F). In a different variant called Flex minibody (Fig. 5G), the scFv is fused to the hinge region of IgG1 which in turn is fused to via an additional 10 residues to the CH3 domain (Hu *et al.*, 1996). The dimeric forms of antibody fragment usually do not suffer any loss of apparent antigen binding affinity compared to the parental IgG and show excellent tumor targeting and short half-life (Santimaria *et al.*, 2003).

Improvement of antigen binding affinity

Engineering antibodies for improving their antigen binding affinity has been a very active and probably one of the most extensively studied areas of antibody engineering research. This may be due to the belief that increasing the affinity of an antibody would allow lower doses to elicit a more profound biological activity which in turn would increase the therapeutic window and lower dose related toxicity. In addition, cost can also be reduced. Comparison and analysis of several independent studies now reveal that the relation between antibody affinity and bioactivity is linear only up to a threshold level (Adams *et al.*, 2001). It is dictated to a large extent by the nature of the antigen and the target tissue, the density of the antigen on the target tissue, and the mode of action of the therapeutic antibody. Affinity maturation of antibodies has been aided by the phage display and yeast display due to its simplicity and high throughput effect in screening high affinity variants.

The approaches to improve antibody affinity can be basically divided into two broad categories. One approach is to create very large libraries of randomly mutated CDRs or the entire variable domains and then select for higher affinity variants from this large collection of mutants (Adams *et al.*, 1998; Barbas *et al.*, 1994; Chames *et al.*, 2002; Maynard *et al.*, 2002; McCall *et al.*, 2001; Schier *et al.*, 1996a; 1996b; Yang *et al.*, 1995; Zhang *et al.*, 2004). The large libraries are created by random CDR mutagenesis, chain shuffling, or error prone PCR and DNA shuffling. The other approach is to make small libraries by focused mutagenesis or hot spot mutagenesis mimicking *in vivo* affinity maturation. In this focused approach, every single position in each of the six CDRs or certain discrete spots of the variable domains called hot spots are randomized and high affinity variants are selected. It is a common practice to combine different mutations that lead to small increases in affinity. Often these combinations of different mutations have an additive or synergistic effect and lead to a greater improvement in affinity (Chowdhury, 2003; Chowdhury and Pastan, 1999; Glaser *et al.*, 1992; Hassan *et al.*, 2002; Ho *et al.*, 2005; Kuan *et al.*, 2000; Salvatore *et al.*, 2002; Wu *et al.*, 1998; 1999).

Improvement of effector functions

Therapeutic antibodies work by one of two basic mechanisms. One is by blocking ligand-receptor interaction or by triggering an intracellular signal, such as apoptosis. The action of these antibodies is largely dependent on their antigen binding function and not on their effector functions. The other way therapeutic antibodies work is by recruiting immune system components following antigen binding. The therapeutic efficacy of these antibodies is therefore dependent on their antigen binding ability as well as their ability to trigger effector activity. These effector functions include ADCC and CDC. It is therefore conceivable that engineering antibodies to improve their binding to FcγRs or the complement factors may lead to improvement in their therapeutic efficacy. However, under certain circumstances, abrogating ADCC activity may be desirable, for example, in the treatment of diseases where lysing of target cells can cause release of pro-inflammatory cytokines. In these situations, one may be able to use those IgG variants whose binding to FcγRs (especially IIIA) or to complement factors are greatly reduced.

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Improvement of ADCC

In humans, there are several types of FcγRs present on the effector cells. These include FcγR1 (CD64), FcγRII (CD32), and FcγRIII (CD16). The FcγRII and III have subtypes A and B. The FcγRIIA has two naturally occurring allotypes for position 131, namely Arg^{131} and His^{131}. The FcγRIIIA has three naturally occurring allotypes at position 48 (Leu, His, and Arg) and two at position 158 (Val and Phe). The different allotypes vary in their ability to bind IgGs and in triggering effector activities. The FcγRI, IIA and IIIA and IIIB are called stimulatory receptors and facilitate ADCC or other cellular effector functions while FcγRIIB is an inhibitory receptor and down regulates ADCC (Daeron, 1997). The three receptors also differ in their affinity for IgG. The FcγRI binds monomeric IgG with high affinity ($K_a = 10^{-8}$–10^{-9} M^{-1}), while FcγRII and FcγRIII have lower affinity for monomeric IgG ($K_a \leq 10^{-7}$) and interact effectively only with multimeric immune complexes. Regarding the expression of the different receptors on the effector cells, NK cells which are believed to be the main effector cell in ADCC express only the stimulatory Fcγ RIIIA, while macrophages express the stimulatory FcγRI, IIA, and IIIA but also the inhibitory FcγRIIB, and neutrophils express the stimulatory FcγRI, IIA, and IIIB but also the inhibitory FcγRIIB. Therefore, to improve ADCC activity, one can improve the affinity of IgG Fc for the stimulatory receptors. The interaction of the IgG with the FcγRs depend on the oligosaccharide in the Fc region and on several residues located in the hinge and CH2 regions. Consequently, engineering for improved ADCC activity has involved glycoengineering of IgG Fc and mutating the residues that contribute to FcγR binding.

Glycoengineering An IgG molecule contains two N-linked glycan chains attached to Asn^{297} in each of its heavy chains in the Fc region. It is well known that IgG is produced as a heterogeneous population of gylcoforms in mammalian cells (Fig. 7). Fc glycosylation is important for the interaction with FcγR. This interaction is known to be sensitive to changes in the oligosaccharide structures of the Fc region (Lund *et al.*, 1996; Wright and Morrison, 1998). The oligosaccharide core normally found attached to the human IgG Fc is of the bi-antennary type and consists of Asn^{297}-linked GlcNAc(Fuc)-GlcNAc-Man-(Man-GlcNAc)$_2$. Individual IgG molecules vary with respect to terminal galactose or galactose-sialic acids at one or both of the terminal GlcNAc and/or attachment of a third GlcNAc (bisecting GlcNAc). They also differ with respect to the presence or absence of a fucose residue attached to the GlcNAc that is linked to Asn^{297}.

Recently, it was reported that elimination of the fucose moiety from the core of the Fc N-linked glycan profoundly improved binding to FcγRs and the ADCC activity (Shields *et al.*, 2002; Shinkawa *et al.*, 2003). The

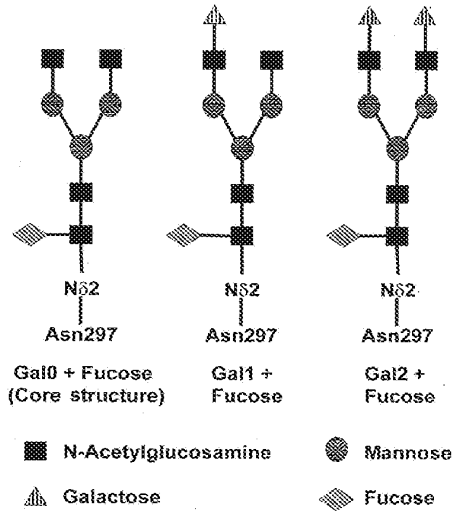


Fig. 7. Schematic representation of immunoglobulin glycoforms.

hypo-fucosylated IgG showed increased binding to FcγRIIIA. In this case, there was at least 42-fold increased binding to the FcγRIIIA(Phe^{158}) allotype and about 19-fold increased binding to the FcγRIIIA(Val^{158}) allotype by the hypo-fucosylated IgG dimers compared to the hyper-fucosylated IgG dimers. In terms of ADCC activity, the hypo-fucosylated IgG showed enhanced activity compared to the parental hyper-fucosylated IgG when PBMCs from individuals with Fcγ RIIIA(Val^{158}/Phe^{158}) and FcγRIIIA(Phe^{158}/Phe^{158}) were used. Therefore, the data suggest that improved binding to FcγRIIIA by the hypo-fucosylated IgG translated into improved ADCC activity.

Protein engineering A number of studies have been done to map the residues in human IgG1 that involve binding to FcγRs. Shields *et al.* (2001) using alanine scanning mutagenesis of all the solvent exposed residues in IgG1 Fc, have extensively mapped the binding site on human IgG1 for FcγRI, II, and III. Based on this study they dissected out the contribution of different residues in the IgG CH2–CH3 domains for binding to FcγRs in general and also in the differential binding to the various subtypes of the FcγRs such as FcγRII and IIIA. From this and other studies (Lazar *et al.*, 2004; Stavenhagen and Vijh, 2004) that focused on screening for IgG1 variants with improved binding to the ADCC stimulatory FcγRIIIA, and decreased binding to the inhibitory receptor, FcγRIIB, several mutants have been reported which had improved ADCC activity. Thus, by grading the contribution of the different residues, Shields *et al.* identified three mutants which when combined together (S298A/E333A/K334A) (EU numbering system; Kabat *et al.*, 1991) showed im-

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proved binding to FcγRIIIA with corresponding improvement in ADCC activity. Additionally, Lazar *et al.* (2004) in a study involving substitution of wild type residues with all different amino acids, identified a mutant S239D/A330L/I332E with greatly improved ADCC activity over the wild type IgG1.

It is interesting to see that the Fc variant, S298A/E333A/K334A, identified by Shields *et al.* (2002) can be combined with de-fucosylation for synergistic ADCC improvement. It should also be kept in mind that the improvement in ADCC activity reported varies to some extent based on the antibody used and also on the types of the cells being employed for assays (Lazar *et al.*, 2004; Shields *et al.*, 2001; Stavenhagen and Vijh, 2004). In addition, the variants discussed in this section have not yet been tested *in vivo* for ADCC improvement. Using transgenic mice expressing human FcγRs will aid the understanding of the *in vivo* activities of these mutations.

Improvement of CDC

CDC is dependent upon binding of two molecules of C1qs to the hinge and CH2 domain of antibodies. Klein *et al.* (1981) had suggested that the hinge region, by modulating the rotational flexibility between the Fab and Fc region, confers on the Fc the ability (or inability) to bind complement and support CDC. A series of studies led by Morrison and co-workers (Sensel *et al.*, 1977; Tao *et al.*, 1991; 1993) employing domain switching and site directed mutagenesis of IgG constant domains, indicated that the CH2 domain had an important contribution to CDC. Based on these and other studies, Idusogie *et al.* (2000) have applied alanine scanning mutagenesis to study the CDC function of Rituxan. This has led to the mapping of the C1q binding epicenter in the CH2 domain of human IgG1. This epicenter involves Asp^{270}, Lys^{322}, Pro^{329}, and Pro^{331} that are spatially close to each other. Idusogie *et al.* (2001) have further identified two residues at the edges of the C1q binding epicenter that could be engineered to increase the C1q binding which consequently improved CDC activity in a human IgG1 format. These residues are Lys^{326} and Glu^{333}, which do not interact with the oligosaccharide chains in CH2. Several different types of residues that do not conform to any particular size, hydrophilicity, and charge were found to be tolerated at each of these two positions. The residues that increased CDC most were Ala, Met, and Trp at position 326, and Ala and Ser at position 333. Combination of some but not all of the single mutations at these positions demonstrated an additive effect on C1q binding and CDC activity.

Introduction of new effector functions

As mentioned above, the two effector functions of therapeutic antibodies are ADCC and CDC. While these effector pathways are important for therapeutic antibody such as Rituxan and probably also for others like Herceptin, there are indications of resistance to CDC and ADCC among cancer cells (Gelderman *et al.*, 2004; Golay *et al.*, 2000; Treon *et al.*, 2001). It is also important to note that ADCC and CDC require the presence of certain minimum level of antigen expression on the target cells and the response is affected by the polymorphic state of FcγRs in the case of ADCC. Therefore, in some therapeutic settings, additional engineering of novel effector functions would be beneficial.

Novel effector activities are provided by conjugating MAbs or their antigen binding domains with diverse classes of molecules, such as cytotoxic drug (for example, calicheamycin conjugated to Mylotarg, approved by FDA), toxin (ricin, Smallshaw *et al.*, 2003), cytokine (IL-2, Neal *et al.*, 2004), enzyme (bacterial carboxyl peptidase, Medzihradszky *et al.*, 2004) in antibody-directed enzyme prodrug therapy, radio-isotopes (^{131}I, ^{90}Y), or even another antibody (anti-activating Fc receptors, Valone *et al.*, 1995a; 1995b) to attract effector cells to the target as in bi-specific antibodies. In these cases, the antibody simply acts as a homing device to carry its payload to the target where the latter precipitates a biologic activity with minimum side effects to non-target tissues.

Altering pharmacokinetics

Increased FcRn binding The plasma half-life of IgG1 is dictated by its binding to FcRn receptor. The site on IgG that is responsible for binding to FcRn has been mapped and well characterized. Hence, the trend in the field of antibody engineering is to mutate the FcRn binding site such that binding to FcRn is increased at pH 6.0 but not at pH 7.4. Dall'Acqua *et al.* (2002) found that major improvement in FcRn binding occurred when mutations were introduced at positions 252, 254, 256, 433, 434, and 436 which are at the interface of the Fc-FcRn binding region. More recently, Hinton *et al.* (2003) discovered two mutations, T250Q and M428L, that caused respectively a 3- and 7-fold increase in FcRn binding and when combined together caused a 28-fold increase in FcRn binding. When tested in rhesus monkeys, the M428L or T250Q/M428L mutant showed about 2-fold increase of plasma half-life compared to the parental molecule. Because these positions are conserved among different human IgG isotypes they proposed that the beneficial effect of these mutations should hold good for other isotypes as well.

PEGylation The huge potential advantage of antibody drugs are somewhat offset by factors such as their high manufacturing cost in mammalian cell lines and limita-

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tions in current world-wide manufacturing capacity. An alternative option is to use *Escherichia coli* for the manufacture of antibody fragments. As discussed earlier, these fragments have exceedingly shorter plasma half-lives compared to whole antibodies. Therefore, development of the methods to increase in the half-lives of antibody fragments is an important area of research. One method is called PEGylation, the process of conjugating polyethylene glycol chains to the antibody fragments. PEGylation of proteins and liposomes has been a time tested and successful technique that offered the advantage of reducing immunogenicity, increasing the plasma half-life, increasing solubility, and reducing protease sensitivity (Chapman, 2002). Therefore, the science of antibody PEGylation has two primary aims which are (a) to preserve the antigen binding activity completely and (b) to link the PEG molecule to the antibody in a stable manner. These are achieved by doing site specific PEGylation using maleimide chemistry. Site specific PEGylation was done by introducing a free cysteine to the end of the hinge region in a Fab (Chapman *et al.*, 1999) or by incorporating the hinge region on the C-terminus of a Fab and scFv (King *et al.*, 1994). In the case of a scFv-immunotoxin, the free cysteine was introduced in the linker between the scFv and the toxin for PEGylation (Tsutsumi *et al.*, 2000). The increase in half-life observed with PEGylated antibody fragments is usually due to a prolongation of the α phase, a phase that represents the redistribution of a molecule in the extravascular environment. It therefore appears that PEGylation slows the redistribution of the molecules from the plasma to the interstitial compartment.

Conclusions

This review briefly updates the different aspects and avenues of antibody engineering that have been attempted to generate therapeutic antibodies and increase their therapeutic efficiencies. While many of these have been tested for their effectiveness *in vivo* and in patients, immunogenicity is a potential risk factor and only clinical trials in humans will evaluate how well humans can tolerate engineered antibodies. Other areas that have not been covered in this review but that have high impact on the success of antibodies as drugs include the improvement of production levels to control the cost of manufacturing and shelf-life of antibody drugs. Antibody drugs for various indications are pursued vigorously and will be clinically used for the treatment of cancer, immune disorders, and infectious diseases.

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