Annex 1

Pfizer Inc's opposition statement against EP 1 720 571 B1 (ALEXION PHARMACEUTICALS, INC.)
"Method of treating hemolytic disease"

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1 Introduction

This constitutes opponent Pfizer Inc's statement under Rule 76 (c) EPC, namely the statement of the extent to which European Patent number EP 1 720 571 B1 ("the Patent") is opposed and the grounds on which the opposition is based as well as an indication of facts, evidence and arguments presented of these grounds.

The following documents are introduced into the proceedings:

	Document	**Publication date**
D1	Hillmen et al, The New England Journal of Medecine 2004;350:552-9	Feb 5, 2004
D2	Medscape Medical News, Dec 13, 2002, Jane Salodof MacNeil	Dec 2002
D3	Hillmen et al, British Journal of Hematology 121 (Suppl. 1) 87-95, 2003, Abstract 257	April 2003
D4	Hillmen et al, Blood. 100(11). November 16, 2002. Abstract No. 154	Nov 2002
D5	Hillmen et al, Journal of The American Society of Hematology 102(11), 2003, Abstract 1858	Nov 2003
D6	Rother et al, Molecular Immunology. 40(2003), p 197, Abstract 54	Sept 2003
D7	Rosse et al, American Society of Hematology – Education program, January 2004 (2004-01), pages 48-62	Jan 2004
D8	Biotechnology News, 17 DEC 2004, 24(30), p.10	Dec 2004
D9	U.S. Patent No. 6,355,245	2002
D10	Thomas et al, Molecular Immunology, Vol. 33, No. 17/18, pages 1389-1401, 1996	1996
D11	Drugs of the Future 2004, 29(7): 673-676	July 2004
D12	Hillmen et al, British Journal of Haematology, 125 (Suppl.1),1-72, Abstract 5	April 2004
	Decision	
	T1979/09	Sept 2011

All the claims of the Patent are opposed.

The grounds on which the Patent is opposed are:
Article 100(c)/Article 123(2) EPC: Added subject-matter
Article 100(a)/Article 54 EPC: Lack of novelty
Article 100(a)/ Article 56 EPC: Lack of inventive step
Article 100(b)/Article 83 EPC: Insufficiency of disclosure

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2- The patent

2.1 Formalities

The Patent was granted on European Patent Application No.05712607.0, which was derived from PCT Patent Application No. PCT/US2005/003225 ("the PCT Application" or "the Application as filed"), which was filed on Feb 03, 2005 ("the Filing Date") and published as WO2005/074607. Priority was claimed from US Patent Application No. 10/771,552 ("Priority Application") filed on Feb 03, 2004 ("Claimed Priority Date").

2.2 The Claims

There are eight claims in the Patent, two of which are independent claims, namely claims 1 and 5. The remaining claims are all dependent, directly or indirectly, on at least one of the independent claims.

3- Requests

It is requested that the Patent be revoked in its entirety.

Oral proceedings are requested if the Patent cannot be revoked on the basis of the written submissions alone. If oral proceedings take place and either the Patentee or any other party chooses to speak in French or German, simultaneous translation into English is requested.

4- Added subject matter (Art 100(c)/Art 123(2) EPC)

According to Art.123(2) EPC a European patent application may not be amended in such a way that it contains subject-matter which extends beyond the content of the application as filed. It is therefore, according to Art.100(c) EPC, a ground of opposition that a European patent contains subject-matter that extends beyond the content of the application as filed. According to the Guidelines for examination (H-IV, 2.2), this legal principle is to be interpreted to mean that “An amendment should be regarded as introducing subject-matter which extends beyond the content of the application as filed, and therefore unallowable, if the overall change in the content of the application (whether by way of addition, alteration or excision) results in the skilled person being presented with information which is not directly and unambiguously derivable from that previously presented by the application, even when account is taken of matter which is implicit to a person skilled in the art”.

The combination of features in claims 1 and 5 of the opposed patent is not directly and unambiguously disclosed by the application as filed, thereby contravening Art.123(2) EPC.

Claims 1 and 5 of the opposed patent were introduced into the application by way of amendment and contain, in combination, the following features:

(1) the use of eculizumab
(2) the treatment of a patient afflicted with paroxysmal nocturnal hemoglobinuria
(3) intravenous administration
(4) administration of 600 mg each week for four weeks

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(5) administration of 900 mg a week later
(6) administration of 900 mg biweekly thereafter
(7) a breakthrough in serum hemolytic activity when the patient is treated under a dosing schedule according to (4) to (6)
(8) administration of 900 mg every 12 days

There is no mention in the claims of the application as filed of any of features (2) to (8), these claims relating to the treatment of hemolytic disease in general terms and not mentioning any features relating to the route of administration or dosage regimen. Therefore, to be allowable according to Art.100(c) EPC, the combination of features (1)-(8) must be directly and unambiguously derivable from the description.

The description discloses feature (2) on page 11, lines 5-6: "The present disclosure relates to a method of treating paroxysmal nocturnal hemoglobinuria ("PNH") and other hemolytic diseases in mammals". Within this broad umbrella, the description contemplates the treatment of a variety of different diseases and symptoms such as abdominal pain, fatigue, dyspnea, insomnia, hemoglobinuria, anemia, erectile dysfunction, dysphagia, thrombosis (page 23, lines 7-17).

As for feature (1), it is disclosed that a large number of compounds of varying pharmacology can be used in this method. These compounds bind to or otherwise block the generation and/or activity of one or more complement components (page 11, lines 24-25). From this broad class, anti-C5 antibodies are described as particularly useful (page 12, lines 1-3) and h5G1.1-mAb (eculizumab) is described as a particularly useful anti-C5 antibody (page 18, lines 12-20).

Regarding feature (3), the selected compound can be administered via a number of routes, one of which is "intravenous infusion by injection" (page 20, lines 4-8). However, the term "intravenous administration" as used in the claim, covers both injection into a vein using a hypodermic needle and the use of a catheter and therefore has a broader meaning than "intravenous infusion by injection".

There is no disclosure in the general description of the invention of any specific dosage or dosage regimen. On the contrary, it is disclosed that the dose selected should vary considerably depending on the compound selected, the manner of administration, the particular symptoms of the patient, the overall health, condition, size and age of the patient, and the judgment of the prescribing physician (page 19, lines 17-23; page 20, lines 1-3). The general teaching of the application, therefore, is that the dose to be administered should be chosen by the physician on a patient-by-patient basis.

In fact, the only basis for features (4)-(8) comes from the specific Example of the application as filed presented on pages 32-39. In this specific, detailed example, eleven patients from a particular patient group (PNH patients that were transfusion-dependent and hemolytic) were treated weekly with a 600 mg intravenous infusion of eculizumab for four weeks, then with 900 mg eculizumab one week later (by an unspecified route) and then with 900 mg eculizumab on a bi-weekly basis (page 32, lines 5-16; also by an unspecified route). Two of the patients exhibited breakthrough in serum hemolytic activity during this biweekly phase of the treatment which was controlled by reducing the dosing interval from fourteen to twelve days (page 35, lines 8-17).

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There is therefore no unambiguous disclosure of the claimed invention in the description. Rather, the features of the claim are drawn separately and variously from different parts of the disclosure, some of which are contradictory, requiring the creation of intermediate generalisations and/or selections.

The subject-matter of claim 1 is not directly and unambiguously derivable from the general part of the description since:

(a) It is necessary to select a pharmacological class (anti-C5 antibody) from a list of such classes (compounds that bind to or otherwise block the generation and/or activity of one or more complement components); a specific compound (eculizumab) from a list of compounds within that class; the disease PNH as the hemolytic disease to be treated from a list of several diseases; and a route of administration (intravenous) from several possible routes.

(b) There is no disclosure of "intravenous administration" in general terms, only the disclosure of "intravenous infusion by injection".

(c) There is no disclosure of any specific dosage regimen, only the disclosure that dosage will vary depending on the exact clinical situation to be addressed.

The subject-matter of claim 1 is not directly and unambiguously derivable from the Example since:

(a) the example is a specific disclosure that contains several other essential features that are missing from claim 1. In particular, the patients in this study were transfusion-dependent and hemolytic – this is a specific patient sub-group and the invention has not been shown to work in other sub-groups. Claim 1 therefore represents a generalisation of the actual teaching of the Example. Moreover, the specific meaning ascribed to the term "transfusion-dependent" on page 32, lines 7-9 should be specified in the claim. Likewise, the infusion time of "approximately 30 minutes" (page 32, lines 12-14) is not specified in the claim, despite being an essential feature of the Example. Finally, the breakthrough occurred in the extension study between weeks 12 and 64 (page 32 lines 17-20; page 35, lines 8-9) whereas the claim is not so limited and covers treatment regimens where the shorter 12 day treatment cycle is initiated in the initial 12 week acute phase of treatment.

(b) In the Example the patients received "a weekly 600 mg intravenous infusion" of eculizumab which implies (i) the drug was administered at time points one week apart and (ii) a catheter-based intravenous infusion was used to administer the drug. The corresponding features of the claim are "intravenous administration" which covers the use of a catheter and a direct injection and administration of "600 mg each week for four weeks" which implies that the drug was administered once within each of four successive calendar weeks but not necessarily at weekly intervals.

(c) In the Example, the route by which the drug was administered following the initial four week dosing is not specified whereas in the claim the route is specified as the intravenous route.

It is furthermore not appropriate to combine features from the general part of the description and the Example since:

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(a) This would create an intermediate generalisation – the features of the Example are detailed and specific and associated with each other and to take one or more out of this narrow context and combine it with features of the invention described in general terms would create embodiments of the invention which were not contemplated in the application as filed.

(b) The general part of the description and the Example are not compatible teachings in that the general part of the description clearly contemplates a treatment in which the dosage is selected on a patient-by-patient basis according to the exact clinical situation presented whereas the Example teaches that the same, specific dosage should be administered to each patient regardless of the particular symptoms, overall health, condition, size and age of the patient.

Therefore, claims 1 and 5 of the patent include subject matter which is not directly and unambiguously derivable from the description or claims of the application as filed and are not allowable according to Art.123(2) and Art.100(c) EPC. The dependent claims, which all contain the features of claims 1 and 5, also contravene these provisions.

5- Lack of novelty (Article 100(a)/Art 54 EPC)

5.1 – The subject matter of claim 1 and 5 is not entitled to the priority

According to Art.87(1) EPC, the priority of an earlier application may be claimed if it discloses "the same invention". According to the Guidelines for Examination (F-VI, 1.3) and G2/98, this is to be interpreted to mean that the subject matter of a claim in a European patent may enjoy the priority of a previous application only if the skilled person can derive the subject-matter of the claim directly and unambiguously, using common general knowledge, from the previous application as a whole.

The combination of features in claims 1 and 5 of the opposed patent is not directly and unambiguously disclosed in the priority application (US patent application No. 10/771,552 filed on 03 February 2004), in contravention of Art.87(1). The claims are therefore not entitled to the priority date.

Claims 1 and 5 of the opposed patent contain, in combination, the following features:

(1) the use of eculizumab
(2) the treatment of a patient afflicted with paroxysmal nocturnal hemoglobinuria
(3) intravenous administration
(4) administration of 600 mg each week for four weeks
(5) administration of 900 mg a week later
(6) administration of 900 mg biweekly thereafter
(7) a breakthrough in serum hemolytic activity when the patient is treated under a dosing schedule according to (4) to (6)
(8) administration of 900 mg every 12 days

There is no mention in the claims of the priority application of any of features (2) to (8), these claims relating to the treatment of hemolytic disease in general terms and not mentioning any

features relating to the route of administration or dosage regimen. Therefore, for the priority claim to be valid, the combination of features (1)-(8) must be directly and unambiguously derivable from the description.

The description of the priority application discloses feature (2) on page 4, lines 13-14: "Paroxysmal nocturnal hemoglobinuria ("PNH") and other hemolytic diseases are treated in accordance with this disclosure". Within this broad umbrella, the description contemplates the treatment of a variety of different diseases and symptoms such as hemoglobinuria, anemia, fatigue, low red blood cell count, erectile dysfunction, dysphagia and thrombosis (page 22, lines 12-17).

As for feature (1), it is disclosed that a large number of compounds of varying pharmacology can be used in this method. These compounds bind to or otherwise block the generation and/or activity of one or more complement components (page 11, lines 7-9). From this broad class, anti-C5 antibodies are described as particularly useful (page 11, lines 9-11) and h5G1.1-mAb (eculizumab) is described as a particularly useful anti-C5 antibody (page 17, line 23 – page 18, line 8).

Regarding feature (3), the selected compound can be administered via a number of routes, one of which is "intravenous infusion by injection" (page 19, lines 15-18). However, the term "intravenous administration" as used in the claim, covers both injection into a vein using a hypodermic needle and the use of a catheter and therefore has a broader meaning than "intravenous infusion by injection".

There is no disclosure in the general description in the priority application of any specific dosage or dosage regimen. On the contrary, it is disclosed that the dose selected should vary considerably depending on the manner of administration, the particular symptoms of the patient, the overall health, condition, size and age of the patient, and the judgement of the prescribing physician (page 19, lines 12-14). The general teaching of the application, therefore, is that the dose to be administered should be chosen by the physician on a patient-by-patient basis.

In fact, the only basis for features (4)-(8) comes from the specific Example of the invention presented on pages 30-35. In this specific, detailed example, eleven patients from a particular patient group (PNH patients that were transfusion-dependent and hemolytic) were treated weekly with a 600 mg intravenous infusion of eculizumab for four weeks, then with 900 mg eculizumab one week later (no route of administration specified) and then with 900 mg eculizumab on a bi-weekly basis (page 30, lines 6-16; no route of administration specified). One of the patients exhibited breakthrough in serum hemolytic activity during this biweekly phase of the treatment which was controlled by reducing the dosing interval from fourteen to twelve days (page 32, lines 13-19).

There is therefore no unambiguous disclosure of the claimed invention in the description. Rather, the features of the claim are drawn separately and variously from different parts of the disclosure, some of which are contradictory, requiring the creation of intermediate generalisations and/or selections.

The subject-matter of claim 1 is not directly and unambiguously derivable from the general part of the description since:

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(a) It is necessary to select a pharmacological class (anti-C5 antibody) from a list of such classes (compounds that bind to or otherwise block the generation and/or activity of one or more complement components); a specific compound (eculizumab) from a list of compounds within that class; the disease PNH as the hemolytic disease to be treated from a list of several diseases; and a route of administration (intravenous) from several possible routes.

(b) There is no disclosure of "intravenous administration" in general terms, only the disclosure of "intravenous infusion by injection".

(c) There is no disclosure of any specific dosage regimen, only the disclosure that dosage will vary depending on the exact clinical situation to be addressed.

The subject-matter of claim 1 is not directly and unambiguously derivable from the Example since:

(a) the example is a specific disclosure that contains several other essential features that are missing from claim 1. In particular, the patients in this study were transfusion-dependent and hemolytic – this is a specific patient sub-group and the invention has not been shown to work in other sub-groups. Claim 1 therefore represents a generalisation of the actual teaching of the Example. Moreover, the specific meaning ascribed to the term "transfusion-dependent" on page 30, lines 8-9 should be specified in the claim. Likewise, the infusion time of "approximately 30 minutes" (page 30, lines 13-14) is not specified in the claim, despite being an essential feature of the Example.

(b) In the Example the patients received "a weekly 600 mg intravenous infusion" of eculizumab which implies (i) the drug was administered at time points one week apart and (ii) a catheter-based intravenous infusion was used to administer the drug. The corresponding features of the claim are "intravenous administration" which covers the use of a catheter and a direct injection and administration of "600 mg each week for four weeks" which implies that the drug was administered once within each of four successive calendar weeks but not necessarily at weekly intervals.

(c) In the Example, the route by which the drug was administered following the initial four week dosing is not specified whereas in the claim the route is specified as the intravenous route.

It is furthermore not appropriate to combine features from the general part of the description and the Example since:

(a) This would create an intermediate generalisation – the features of the Example are detailed and specific and associated with each other and to take one or more out of this narrow context and combine it with features of the invention described in general terms would create embodiments of the invention which were not contemplated in the application as filed.

(b) The general part of the description and the Example are not compatible teachings in that the general part of the description clearly contemplates a treatment in which the dosage is selected on a patient-by-patient basis according to the exact clinical situation presented whereas the Example teaches that the same, specific dosage

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should be administered to each patient regardless of the particular symptoms, overall health, condition, size and age of the patient.

Therefore, claims 1 and 5 of the patent include subject matter which is not directly and unambiguously derivable from the priority application and are not entitled to the priority date. The validity of these claims is consequently to be considered as of the filing date of 03 February 2005.

5.2 – Features of claims 1 and 5 as granted

Claim 1 as granted contains the following features:

(1) the use of eculizumab
(2) the treatment of a patient afflicted with paroxysmal nocturnal hemoglobinuria
(3) intravenous administration
(4) administration of 600 mg each week for four weeks
(5) administration of 900 mg a week later
(6) administration of 900 mg biweekly thereafter
(7) a breakthrough in serum hemolytic activity when the patient is treated under a dosing schedule according to (4) to (6)
(8) administration of 900 mg every 12 days

The same numbering can be applied to claim 5 which comprises the same features as claim 1. The above numbering of the features of claims 1 and 5 will be used in the following sections.

5.3 – D1 (Hillmen et Al, 2004) anticipates the subject matter of claims 1 to 8

D1 published on Feb 5, 2004 and is therefore part of the state of the art under Art 54(2) EPC against claims which are not entitled to the priority date.

- Claims 1 and 5

On page 552, paragraph entitled “Methods”, D1 describes a trial where 11 patients with PNH **(2)** received infusions **(3)** of Eculizumab **(1)** (600mg) every week for 4 weeks **(4)**, followed one week later by a 900-mg dose **(5)** and then by 900 mg every other week **(6)** through week 12.

On page 555, last sentence to page 556, first sentence, D1 describes one patient from the trial in whom eculizumab level fell below 35µg per milliliter at week 12, hemolytic activity returned **(7)** and lactate deshydrogenase levels increased transiently. The dosing frequency was increased from 900 mg every 14 days to 900 mg every 12 days **(8)**, reestablishing complete complement blockade during the ongoing 12-month extension study (data not shown).

Therefore, D1 anticipates claims 1 and 5 which lack novelty.

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- Claims 2, 3, 6 and 7

On page 554, third full sentence, D1 describes that patients from the trial disclosed above "who were taking stable doses of immunosuppressive drugs (e.g., cyclosporine), warfarin, and iron supplements were permitted to continue them".

Therefore, D1 anticipates claims 2, 3, 6 and 7 which lack novelty.

- Claims 4 and 8

On page 555, figure 3A, the percentage of type III erythrocytes in patients from the above mentioned study is greater than 10% for all patients.

Therefore, D1 anticipates claims 4 and 8 which lack novelty.

6- Lack of Inventive Step (Article 100(a)/ Art 56 EPC)

As argued above the opponent is of the opinion that the subject matter of all claims lacks novelty. Should the Opposition Division (OD) be of a different opinion, the opponent reserves the right to use D1 cited in the novelty section for assessment of inventive step.

D7 (see page 53, last 5 lines first column and first 4 lines of second column), D8 (see 1st paragraph), D11 (see page 675, sentence bridging columns and first whole sentence in second column) and D12 (second column, lines 20 to 29) also disclose reduction of dosing interval of Eculizumab to prevent breakthrough of serum hemolytic activity and could also be used for assessment of inventive step.

As detailed below, in the unlikely case that the OD would consider the subject matter of claims entitled to the priority, we submit that none of the granted claims is inventive over the prior art published before the priority date.

6.1 Use of Eculizumab for the treatment of PNH was known at the priority date.

D2 published on Dec 13, 2002 and is therefore part of the state of the art under Art 54(2) EPC against all claims.[1]

D2 is an article discussing results from clinical trials conducted with Eculizumab. As mentioned in the 2nd paragraph of D2, 11 patients were administered Eculizumab for 3 months. D2 (paragraph 12 – emphasis added) further discloses, that "the PNH trial was conducted with Alexion support at two sites in the U.K. Patients received 600 mg of eculizumab each week for four weeks, after which the dose was switched to 900 mg every two weeks."

D3 (Hillmen et al.) is an abstract published in the British Journal of Haematology on April 9, 2003 and is therefore part of the state of the art under Art 54(2) EPC against all claims. D3 discloses that (emphasis added) "Eleven transfusion-dependent PNH patients have

[1] The Examiner confirmed that D2 is prior art in Section 2 of the Communication dated 21 September 2010 in prosecution

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completed a 3-month pilot study receiving 600 mg Eculizumab by intravenous infusion over 30 min weekly for four doses and then 900 mg every other week."

D4 (Hillmen et al) is an abstract published on Nov 16, 2002 and is therefore part of the state of the art under Art 54(2) EPC against all claims. D4 discloses that "Ten transfusion-dependent PNH patients have been entered into an ongoing open label Pilot study of eculizumab and all patients will have completed the study by November 2002. Patients receive 600 mg eculizumab by intravenous infusion over 30 minutes weekly for 4 doses and then 900 mg every other week".

D6 (Rother et Al) is an abstract published in Sept 2003 and is therefore part of the state of the art under Art 54(2) EPC against all claims. D6 discloses that "Eleven transfusion dependent PNH patients have completed an open-label pilot study of eculizumab. Patients received 600 mg eculizumab by intravenous infusion over 30 min weekly for four doses, followed by 900 mg 1 week later, and then 900 mg every other week for the remainder of the trial."

It was therefore very well established at the priority date that Eculizumab could be used for treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein eculizumab is to be administered intravenously to the patient under the following dosing schedule: 600 mg each week for four weeks; 900 mg 1 week later and 900 mg on a biweekly basis.

6.2 Reducing the dosing interval to prevent potential breakthrough in patients treated with 900mg Eculizumab biweekly was known before the priority date.

D5 (Hillmen et al) is an abstract published on Nov 16 2003 and is therefore part of the state of the art under Art 54(2) EPC against all claims.

D5 relates to the results of the extension of the study disclosed in D4. On lines 5 to 8, D5 discloses that,

> *"We previously reported that eculizumab was very effective at acutely controlling intravascular hemolysis in a 3-month Pilot study of transfusion-dependent PNH patients. We now report that all 11 patients from the initial study entered an extension trial of 900mg eculizumab by i.v. infusion over 30 minutes every other week for one year."*

The pilot study mentioned in the above paragraph is the pilot study discussed lines 8 to 10 of D4. This is consistent with the disclosure of D4, which also mentions that the patients who have completed the pilot study have all entered into an extension study which results are reported in D5.

Therefore the patients from the study discussed in D5 have received 600 mg eculizumab by intravenous infusion over 30 minutes weekly for 4 doses and then 900 mg every other week during the pilot study disclosed in detail in D4. Then they entered into the extension study and continued to receive 900 mg eculizumab by i.v. infusion over 30 minutes every other week (see D5, l.7-8).

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On page 510a, 1st col., l.8-12, D5 discloses that

> *"Two patients experienced transient breakthroughs in complement activity associated with increased LDH, hemoglobinuria and mild symptoms, these resolved immediately upon next dose of eculizumab and further breakthroughs were prevented by a slight decrease in the dosing interval."*

It was therefore very well established at the priority date that breakthroughs in serum hemolytic activity observed in patients afflicted with PNH and treated with Eculizumab under a dosing schedule according to items (i) to (iii) of claim 1 as granted could be prevented by slightly decreasing the dosing interval.

The difference between the subject matter of claim 1 and the disclosure of D5 is that the reduced dosing interval is 12 days.

The effect of this difference is to prevent further breakthrough in serum hemolytic activity in patients which exhibit such breakthrough when treated under a dosing schedule according to items (i) to (iii) of claim 1 as granted.

The objective technical problem to be solved is to identify a reduced interval allowing prevention of further breakthrough in serum hemolytic activity in said patients.

The man skilled in the art, starting from D5 and aiming at reducing the interval between two 900mg doses would have tried different intervals shorter than 14 days and would have determined very easily that a 12 days interval is suitable for preventing further breakthrough in serum hemolytic activity in patients exhibiting breakthrough when treated under a dosing schedule according to items (i) to (iii) of claim 1 as granted. As shown in figure 5 of the patent, this is exactly what has been done during the trial described in the examples of the patent. Figure 5 shows the effect of the dosing interval on hemoglobinuria in one of the two patients exhibiting breakthrough of hemolysis activity. When Eculizumab is administered every 14 days, hemoglobinuria is observed before the next dose is administered (see Fig.5 urine grade at day 13). The dosing interval was reduced to 13 days and hemoglobinuria was still observed on the last day of the interval (see Fig.5 urine grade at day 41). Therefore, the dosing interval was reduced to 12 days and no further hemoglobinuria event is observed in Fig.5.

The skilled person, observing a breakthrough in hemolytic activity in a patient administered with 900mg every 14 days, would have applied the teaching of D5 and reduced the dosing interval until it reaches the 12 days dosing interval such that further breakthrough were prevented. Indeed such a reduction in dosing interval represents a routine modification.

We make reference to T1979/09. Section 7 of the reasons of the decision concern an auxiliary request which included a dosage regimen. Reasons 7.3.1 states that:

> *"It is well known that in designing dosage regimen the two major parameters that can be adjusted are the dose itself, i.e. the quantity of drug administered, and the dosing frequency, i.e. the time interval between doses."*

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Further, in Reasons 7.3.2 the Board stated that:

> *"The board considers that it is within the competence of the skilled person to find out the optimal dosage regime. Consequently, the replacement of a dosage regime by another dosage regime for the same purpose is considered to be a matter of routine experimentation and cannot be seen as involving an inventive step."*

This decision is applicable to the present situation - the use of the defined dosage schedule would have been considered routine to the skilled person in view of the prior art and faced with the technical problem.

Therefore claim 1 lack inventive step over D5 alone or in combination with any one of D2, D3, D4 or D6, or over any of D2, D3, D4 or D6. Claim 5 which relates to the same features also lack inventive step for the same reasons.

The additional features of dependant claims 2 to 4 and 5 to 8 do not confer inventive step the claimed use.

7- Insufficiency of disclosure (Art 100(b)/Art 83 EPC)

7.1 Claims 1 to 8 contravene Article 83 because they lack essential features for the alleged technical effect

Eculizumab is an essential feature of claims 1 to 8. The structure of Eculizumab is not disclosed in the application as filed. On page 8, lines 8-9, the patent discloses that h5G1.1-mAb is currently undergoing clinical trials under the tradename Eculizumab. On the same page, lines 5 to 8, the patent further mentions that methods for the preparation of h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1 are described in U.S. Patent No. 6,355,245 (D9) and "Inhibition of Complement Activity by Humanized Anti-C5 Antibody and Single Chain Fv", Thomas et al., Molecular Immunology, Vol. 33, No. 17/18, pages 1389-1401, 1996 (D10).

However, as detailed below, the complete sequence of Eculizumab or h5G1.1 mAb is not disclosed in these two documents.

D9 is a patent assigned to Alexion Pharmaceutical, Inc which relates to C-5 specific antibodies for the treatment of inflammatory diseases.

In example 7, D9 discloses a mouse monoclonal antibody 5G1.1. In example 11 (columns 42 to 44), D9 lists a number of humanized, chimeric and non humanized scFv, Fd and light chains. D9 does not comprise any reference to Eculizumab or h5G1.1 mAb nor does it disclose the sequence of a full length humanized antibody.

D10 is a publication from Alexion Pharmaceuticals relating to humanized anti-C5 antibody and scFv. D10 discloses the cloning of the variable region genes of the mouse mAb 5G1.1 (see page 1390), the humanization of 5G1.1 variable regions and the construction and expression of various Fab and scFv (see page 1391). The sequences of humanized variable regions are provided in Figure 1. The construction of a humanized antibody h5G1.1 called

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h5G1.1 HuG4 is also discussed on page 1396 (paragraph bridging col.1 and 2) and on page 1399, col.2. However, the exact sequence of the humanized antibody is not disclosed in D10.

In view of the above, none of the patent, D9 or D10 provides a detailed description of the structure of h5G1.1 mAb or Eculizumab.

Therefore, the man skilled in the art would not be able to carry out the invention as he would not be able to prepare the specific antibody Eculizumab, which is an essential feature of the claims 1 to 8.

Therefore claims 1 to 8 are not sufficiently disclosed in the sense of Article 83 EPC.

7.2 Claims 1 to 8 contravene Article 83 because the invention is not disclosed in a manner sufficiently clear and complete.

Claim 1 as granted comprises the feature that the "patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii)".

The term "breakthrough in serum hemolytic activity" is neither defined in claim 1 nor in the general disclosure of the application as filed. This term is used in the example section (see page 35, lines 9-10) and in particular in connection with two patients who did not sustain levels of Eculizumab necessary to consistently block complement. Again, no definition of this term is provided in the example. On page 35, lines 11 to 15, the application as filed discloses that "breakthrough of complement blockade resulted in hemoglobuniria, dysphagia and increased LDH and AST which correlated with the return of the hemolytic activity". These events (hemoglobinuria, dysphagia and increased LDH and AST) seem to be associated to some extent with serum hemolytic activity. However, it is not clear if one, two, three or four of these events are necessary and/or sufficient to characterize a breakthrough according to claim 1.

Claim 1 does not provide any indication relative to the characterization of "breakthrough in serum hemolytic activity". In the absence of additional guidance from the description, the skilled person would not be able to determine when a patient exhibits breakthrough in serum hemolytic activity and as a consequence would not be able to determine if and when the dosing interval should be reduced to 12 days.

Therefore, the skilled person would not be able to carry out the subject matter of claims 1 to 8 which therefore contravene Art.83 EPC.

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EUROPEAN PATENT OFFICE
Erhardtstrasse 27
D-80298 Munich
Germany

VIA EPOLINE- Form 2300E

Paris, 12 March, 2013

OUR REF.: SR-01675
THP/IC

Re: European Patent No 1 720 571 B1 (ALEXION PHARMACEUTICALS, INC.)
Application No. 05712607.0
Opponent: PFIZER INC.

Dear Sirs,

In the name of Pfizer Inc., a Notice of Opposition is herewith submitted against the European Patent EP1720571 B1 (ALEXION PHARMACEUTICALS, INC.) entitled "Method of treating hemolytic disease".

It is requested that EP1720571 B1 be revoked in its entirety.

Statement under Rule 76 (c) EPC is attached hereto (Annex 1).

The fee for filing the opposition is paid by debit from deposit account with the EPO No. 28050037.

Yours faithfully,

Thomas PRINGOT
European Patent Attorney
Association Pfizer – No. 462
Phone: (33) 1 58 07 43 69 / Fax: (33) 1 70 70 72 37
Email: thomas.pringot@pfizer.com

Encls: - opposition statement (13 pages)
- cited documents D1-D12
- Decision T1979/09
- EPO Form 2300E

Samsung Bioepis Exhibit No. 1027
Page 014

Europäisches Patentamt
European Patent Office
Office européen des brevets

Notice of opposition to a European patent

I. Patent opposed

Patent No.	EP1720571
Application No.	EP05712607.0
Date of mention of the grant in the European Patent Bulletin (Art. 97(3), Art. 99(1) EPC)	13 June 2012
Title of the invention	METHOD OF TREATING HEMOLYTIC DISEASE

II. Proprietor of the patent

first named in the patent specification	ALEXION PHARMACEUTICALS, INC.
Opponent's or representative's reference	SR-01675

III. Opponent

Name	Pfizer Inc.
Address:	235 East 42nd Street NEW YORK NY New York 10017 United States of America
State of residence or of principal place of business	United States of America

Multiple opponents (see additional sheet) ☐

IV. Authorisation

1. Representative

	Pfizer	
Department:	European Patent Department	
Association:	462	
Address of place of business	23-25 avenue du Docteur Lannelongue 75668 PARIS Cedex 14 France	
Telephone/Fax	+33 1 58 07 43 52	+33 1 70 70 72 37

Multiple representatives (see additional sheet) ☐

Samsung Bioepis Exhibit No. 1027
Page 015

Authorisation(s)

is/are enclosed ☐

has/have been registered under No. ☒ 35

V. Opposition is filed against

the patent as a whole ☒

claim(s) No(s).

VI. Grounds for opposition:

Opposition is based on the following grounds:

(a) the subject-matter of the European patent opposed is not patentable (Art. 100(a) EPC) because:

• it is not new (Art. 52(1); Art. 54 EPC) ☒

• it does not involve an inventive step (Art. 52(1); Art. 56 EPC) ☒

• patentability is excluded on other grounds, namely articles ☐

(b) the patent opposed does not disclose the invention in a manner sufficiently clear and complete for it to be carried out by a person skilled in the art (Art. 100(b) EPC; see Art. 83 EPC). ☒

(c) the subject-matter of the patent opposed extends beyond the content of the application/of the earlier application as filed (Art. 100(c) EPC, see Art. 123(2) EPC). ☒

VII. Facts (Rule 76(2)(c) EPC)

presented in support of the opposition are submitted herewith on an attached document ☒

VIII. Other requests:

• It is requested that the Patent be revoked in its entirety.
Oral proceedings are requested if the Patent cannot be revoked on the basis of the written submissions alone. If oral proceedings take place and either the Patentee or any other party chooses to speak in French or German, simultaneous translation into English is requested.

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IX. Evidence presented

- Other evidence	T1979/09 original file name: T-1979-09.pdf attached as: Other-evidence-1.pdf
D1 Non-patent literature - article	Hillmen et al., "Effect of Eculizumab on Hemolysis and Transfusion Requirements in Patients with Paroxysmal Nocturnal Hemoglobinuria" The New England Journal of Medicine, Vol. 350(6), No. 6, 05 February 2004 particular relevance: pages 552-559 original file name: D1 - Hillmen New England.pdf attached as: Published-Evidence-1.pdf
D10 Non-patent literature - article	Thomas et al., "Inhibition of complement activity by humanized anti-C5 antibody and single-chain Fv." Molecular Immunology, Vol. 33, 1996 particular relevance: Pages 1389-1401 original file name: D10 Thomas et al 1996.pdf attached as: Published-Evidence-10.pdf
D11 Non-patent literature - article	Harding, "Eculizumab, Humanized Anti-C5 Monoclonal Antibody" Drugs of the Future, Vol. 29(7), 2004 particular relevance: Pages 673-676 original file name: D11 - Harding 2004.pdf attached as: Published-Evidence-11.pdf
D12 Non-patent literature - article	Hillmen et al., "Eculizumab, a C5 complement-blocking antibody, controls haemolysis in paroxysmal nocturnal haemoglobinuria (PNH) with responses maintained over a prolonged period of therapy" British Journal of Haematology, Vol. 125(1), 19 April 2004 particular relevance: Abstract 5 original file name: D12 - Hillmen 2004 BJH.pdf attached as: Published-Evidence-12.pdf
D2 Non-patent literature - article	Salodof MacNeil, Jane, "Eculizumab could be Paroxysmal Nocturnal Hemoglobinuria Breakthrough" Medscape Medical News, 13 December 2002 original file name: D2 - McNeil.pdf attached as: Published-Evidence-2.pdf
D3 Non-patent literature - article	Hillmen et al., "Eculizumab, a C5 complement blocking antibody, is the first therapy to reduce transfusion requirements in patients with paroxysmal nocturnal haemoglobinuria (PHN)" British Journal of Haematology, Vol. 121(1), 09 April 2003 particular relevance: Abstract 257 original file name: D3 - Hillmen - British Journal of Hemato - May 2003.pdf attached as: Published-Evidence-3.pdf
D4 Non-patent literature - article	Hillmen et al., "Eculizumab, a C5 Complement-Blocking Antibody, Abolishes Hemolysis and Renders Hemolytic Patients with Paroxysmal Nocturnal Hemoglobinuria (PNH) Transfusion Independent" Blood, Vol. 100;11, 16 November 2002 particular relevance: Abstract 154 original file name: D4 - Hillmen_Blood-2002.pdf attached as: Published-Evidence-4.pdf

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D5	Non-patent literature - article	Hillmen et al., "Eculizumab, a C5 Complement-Blocking Antibody, Controls Hemolysis in Paroxysmal Nocturnal Hemoglobinuria (PNH) with Responses Maintained over a Prolonged period of Therapy" American Society of Hematology, Vol. 102;11, September 2003 particular relevance: Abstract 1858 original file name: D5 - Hillmen_Blood-102-2003.pdf attached as: Published-Evidence-5.pdf
D6	Non-patent literature - article	Rother et al., "Eculizumab, a C5 complement-blocking antibody, abolishes hemolysis and reduces transfusion dependency in patients with paroxysmal nocturnal hemoglobinuria (PNH)" Molecular Immunology, Vol. 40, September 2003 particular relevance: Abstract 54 original file name: D6 - Rother et al - Molecular Immunology (2003).pdf attached as: Published-Evidence-6.pdf
D7	Non-patent literature - article	Rosse et al., "Immune-mediated Hemolytic Anemia" American Society of Hematology - Education program, January 2004 particular relevance: pages 48-62 original file name: D7 - Rosse (2004).pdf attached as: Published-Evidence-7.pdf
D8	Non-patent literature - article	, "Clinics" Biotechnology News, Vol. 24(30), 17 December 2004 particular relevance: page 10 original file name: D8 - Clinics (2002).pdf attached as: Published-Evidence-8.pdf
D9	Patent document	US6355245 (B1) , 12.03.2002 original file name: D9 US6355245.pdf attached as: Published-Evidence-9.pdf

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X. Payment

Mode of payment	Debit from deposit account

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

Currency:	EUR
Deposit account number:	28050037
Account holder:	Pfizer Limited

Refunds

Any refunds should be made to EPO deposit account:	28050037
Account holder:	Pfizer Limited

Fees	Factor applied	Fee schedule	Amount to be paid
010 Opposition fee	1	745.00	745.00
Total:		EUR	745.00

	A Forms	Details:	System file name:
A-1	Form for notice of opposition		ep-oppo.pdf

	B Attached document files	Original file name:	System file name:
B-1	1. Facts and arguments	2013-03 opposition statement.pdf	OPPO.pdf
B-2	1. Any annexes (other than citation) to an opposition letter - Opposition letter	SR-01675-Notice of oppo.pdf	OTHER-1.pdf

	C Attached evidence files	Original file name:	System file name:
C-1	1. Patent document	D9 US6355245.pdf	Published-Evidence-9.pdf
C-2	1. Non-patent literature - article	D1 - Hillmen New England.pdf	Published-Evidence-1.pdf
C-3	2. Non-patent literature - article	D2 - McNeil.pdf	Published-Evidence-2.pdf
C-4	3. Non-patent literature - article	D3 - Hillmen - British Journal of Hemato - May 2003.pdf	Published-Evidence-3.pdf
C-5	4. Non-patent literature - article	D4 - Hillmen_Blood-2002.pdf	Published-Evidence-4.pdf

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C-6	5. Non-patent literature - article	D5 - Hillmen_Blood-102-2003.pdf	Published-Evidence-5.pdf
C-7	6. Non-patent literature - article	D6 - Rother et al - Molecular Immunology (2003).pdf	Published-Evidence-6.pdf
C-8	7. Non-patent literature - article	D7 - Rosse (2004).pdf	Published-Evidence-7.pdf
C-9	8. Non-patent literature - article	D8 - Clinics (2002).pdf	Published-Evidence-8.pdf
C-10	9. Non-patent literature - article	D10 Thomas et al 1996.pdf	Published-Evidence-10.pdf
C-11	10. Non-patent literature - article	D11 - Harding 2004.pdf	Published-Evidence-11.pdf
C-12	11. Non-patent literature - article	D12 - Hillmen 2004 BJH.pdf	Published-Evidence-12.pdf
C-13	1. Other evidence	T-1979-09.pdf	Other-evidence-1.pdf

Signature of opponent or representative

Place	**Paris**
Date:	**12 March 2013**
Signed by:	**/Thomas PRINGOT/**
Association:	**462**
Capacity:	**(Representative)**

US006355245B1

(12) **United States Patent**
Evans et al.

(10) **Patent No.: US 6,355,245 B1**
(45) **Date of Patent: Mar. 12, 2002**

(54) **C5-SPECIFIC ANTIBODIES FOR THE TREATMENT OF INFLAMMATORY DISEASES**

(75) Inventors: **Mark J. Evans**, Cheshire; **Louis A. Matis**, Southport; **Eileen Elliott Mueller**, East Haven, all of CT (US); **Steven H. Nye**, Mequon, WI (US); **Scott Rollins**, Monroe, CT (US); **Russell P. Rother**; **Jeremy P. Springhorn**, both of Cheshire, CT (US); **Stephen P. Squinto**, Bethany, CT (US); **Thomas C. Thomas**, Madison, CT (US); **James A. Wilkins**, Woodbridge, CT (US)

(73) Assignee: **Alexion Pharmaceuticals, Inc.**, Cheshire, CT (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **08/487,283**

(22) Filed: **Jun. 7, 1995**

Related U.S. Application Data

(63) Continuation of application No. PCT/US95/05688, filed on May 1, 1995, which is a continuation-in-part of application No. 08/236,208, filed on May 2, 1994, now Pat. No. 6,074,642.

(51) **Int. Cl.**[7] **A61K 39/395**; C07K 16/36; C12N 5/12

(52) **U.S. Cl.** ..... **424/145.1**; 424/130.1; 424/133.1; 424/135.1; 424/141.1; 424/145.1; 424/158.1; 424/139.1; 424/130; 530/387.1; 530/387.3; 530/387.9; 530/388.1; 530/388.23; 530/388.25; 530/388.7; 435/326; 435/328; 435/331; 435/33 L; 435/337; 435/343; 435/346

(58) **Field of Search** 530/387.1, 387.3, 530/388.1, 388.7, 387.9, 388.23; 435/69.1, 172.3, 328, 343, 70.21, 325, 331, 332, 346; 424/130.1, 141.1, 139, 133.1, 145.1

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(List continued on next page.)

Primary Examiner—Phillip Gambel

(57) **ABSTRACT**

The use of anti-C5 antibodies, e.g., monoclonal antibodies, to treat glomerulonephritis (GN) is disclosed. The administration of such antibodies at low dosage levels has been found to significantly reduce glomerular inflammation/enlargement and other pathologic conditions associated with GN. Also disclosed are anti-C5 antibodies and anti-C5 antibody-encoding nucleic acid molecules. These antibodies are useful in the treatment of GN and other inflammatory conditions involving pathologic activation of the complement system.

23 Claims, 19 Drawing Sheets

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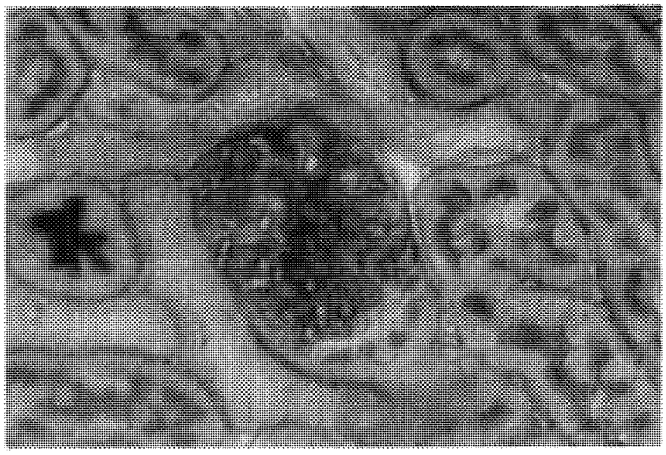


FIG. 1A

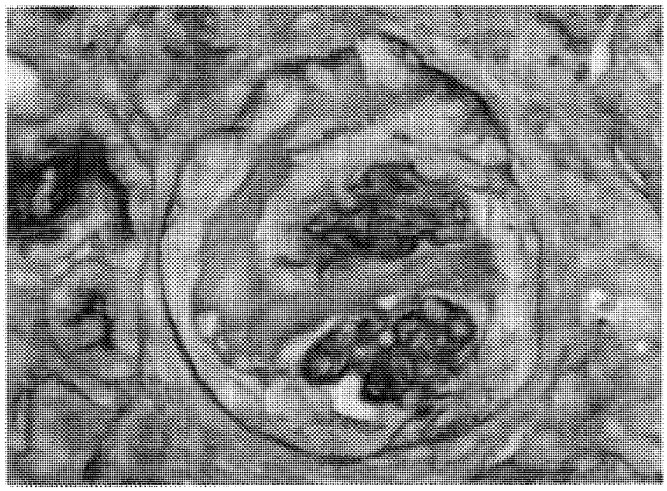


FIG. 1B

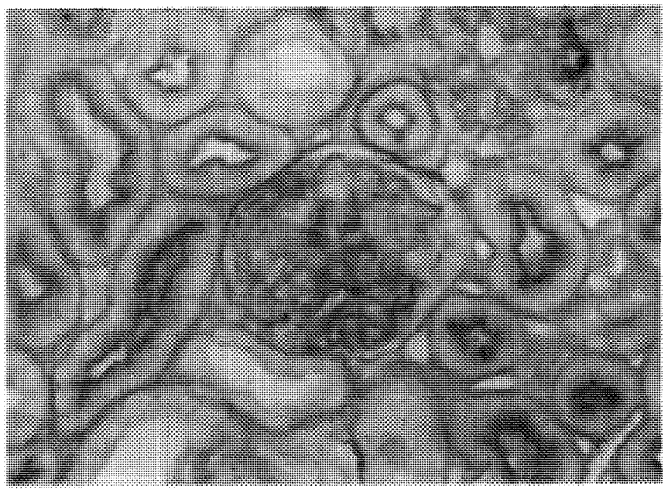


FIG. 1C

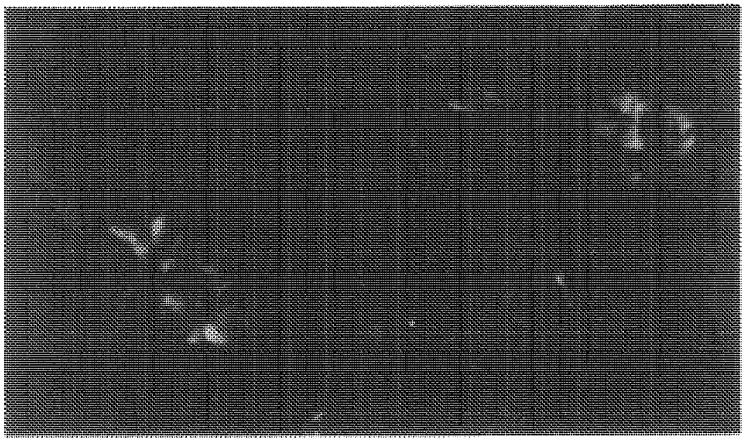


FIG. 2A

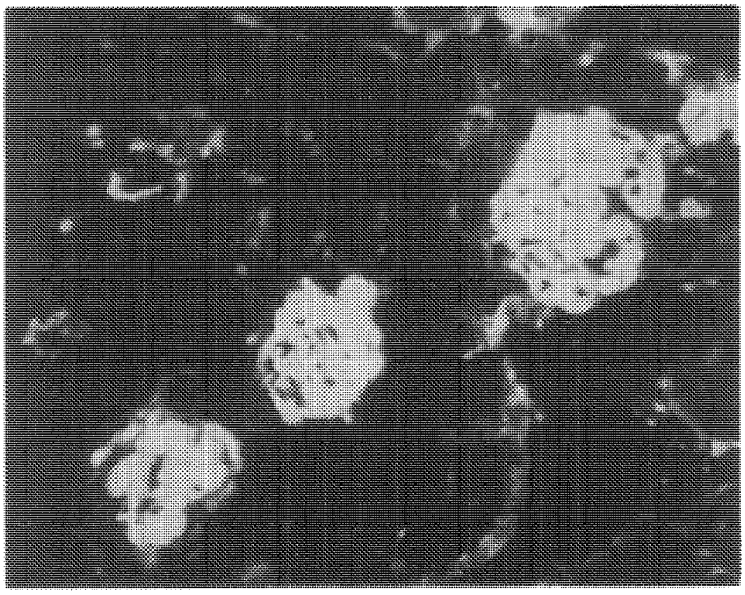


FIG. 2B

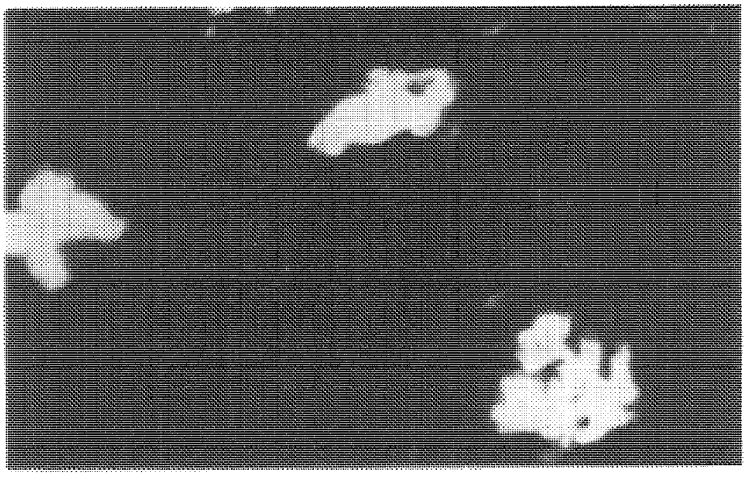


FIG. 2C

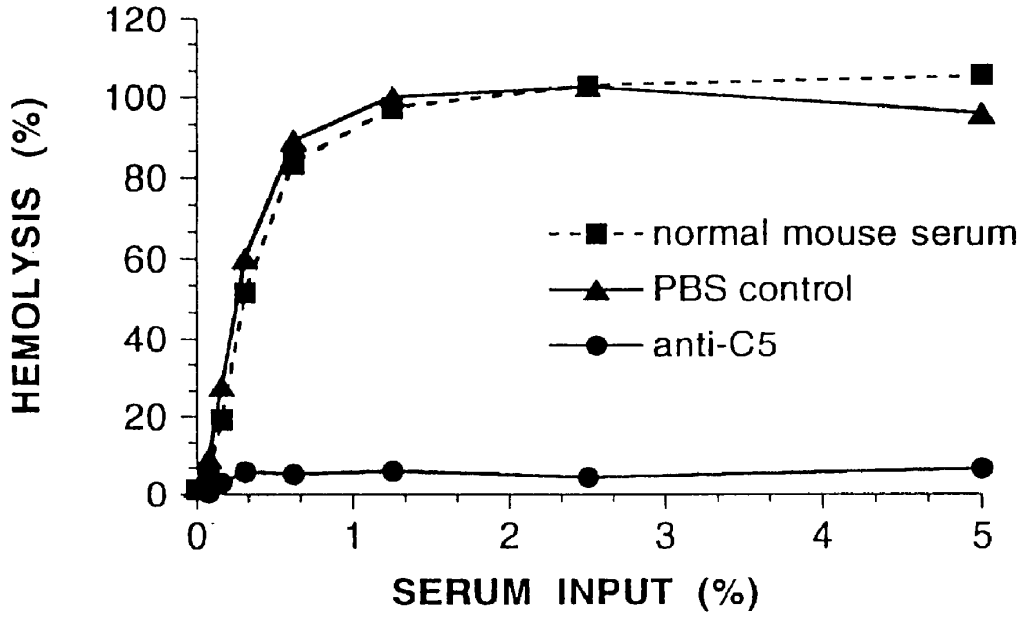

120
100
80
60
40
20
0
HEMOLYSIS (%)
0
1
2
3
4
5
SERUM INPUT (%)
normal mouse serum
PBS control
anti-C5

FIG.3

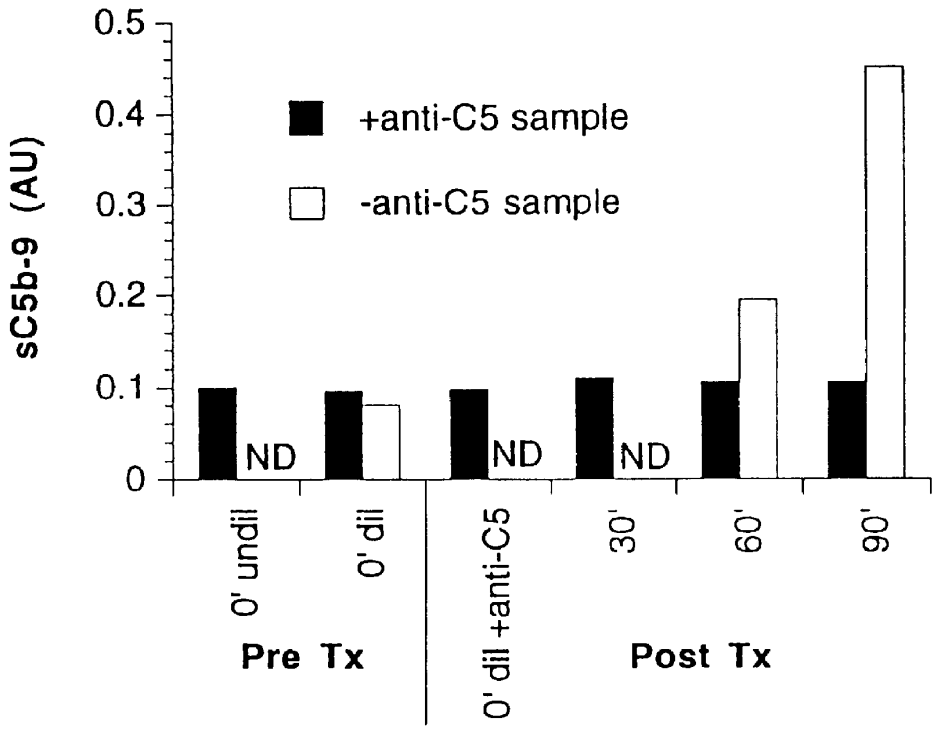

sC5b-9 (AU)
0.5
0.4
0.3
0.2
0.1
0
+anti-C5 sample
-anti-C5 sample
ND
ND
ND
0' undil
0' dil
0' dil +anti-C5
30'
60'
90'
Pre Tx
Post Tx

FIG.4

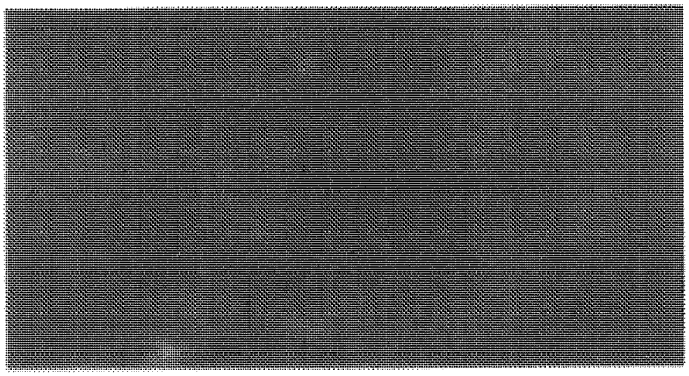


FIG.5A

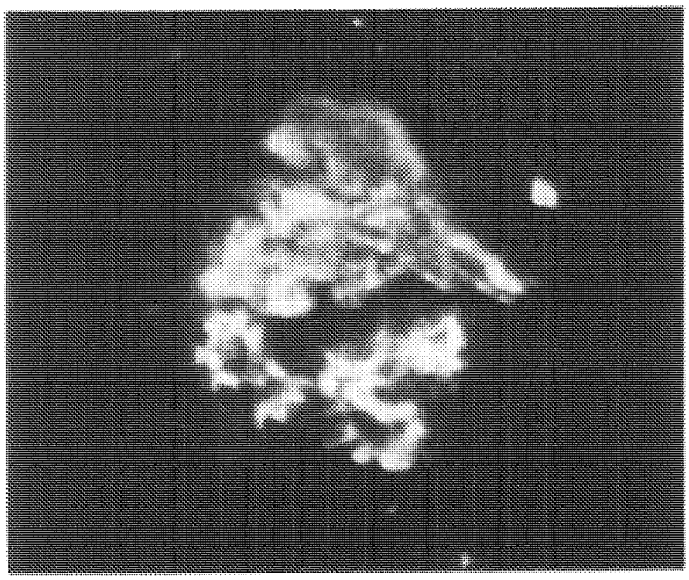


FIG.5B

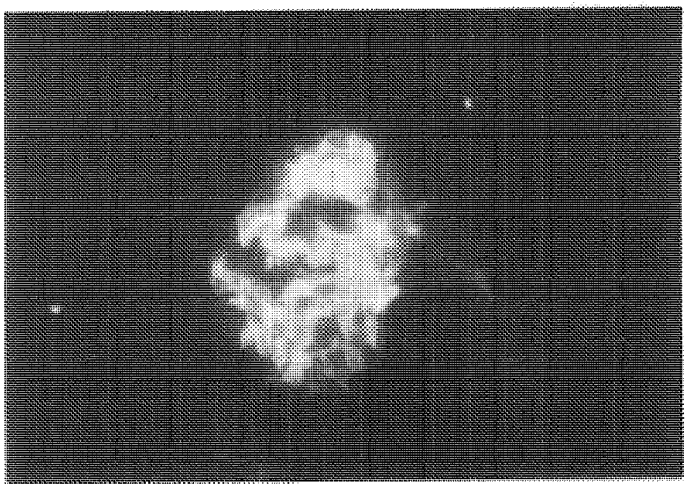


FIG.5C

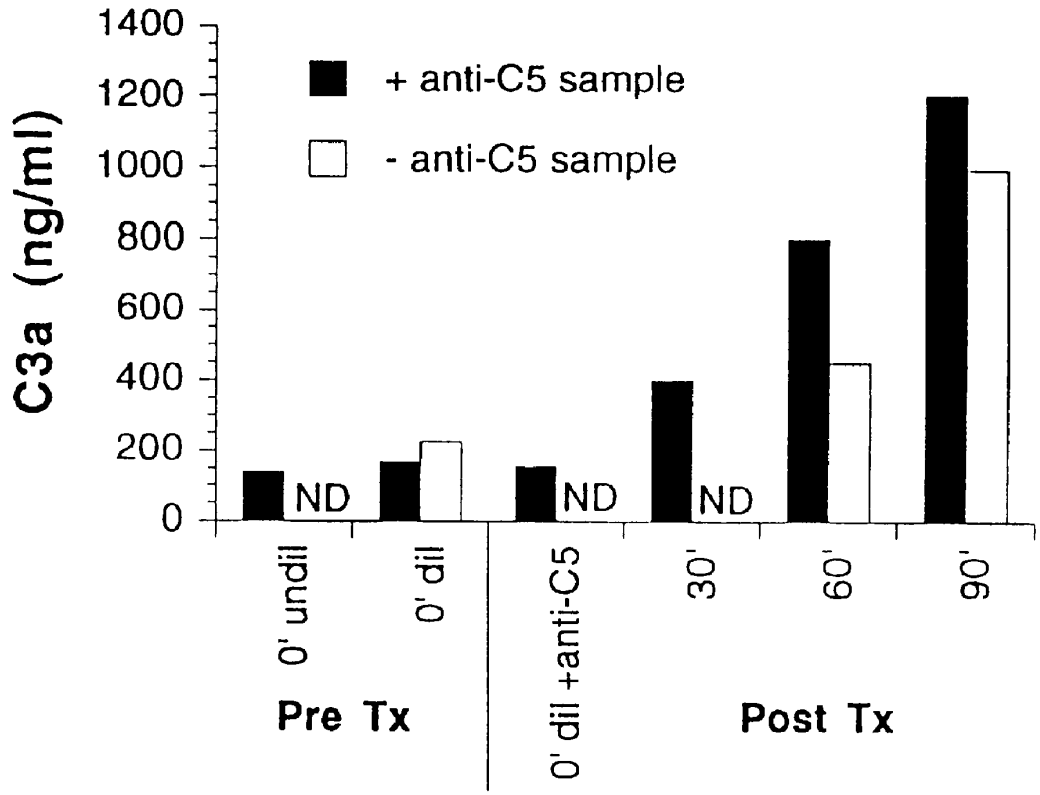

1400
1200
1000
800
600
400
200
0
C3a (ng/ml)
+ anti-C5 sample
- anti-C5 sample
ND
ND
ND
0' undil
0' dil
0' dil +anti-C5
30'
60'
90'
Pre Tx
Post Tx

FIG.6

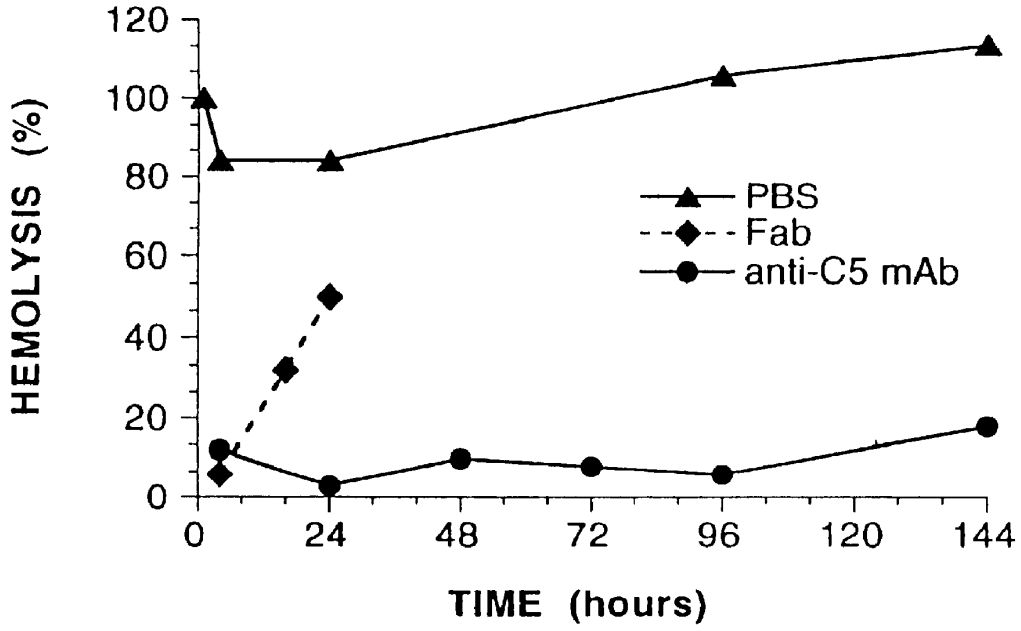

HEMOLYSIS (%)
120
100
80
60
40
20
0
0
24
48
72
96
120
144
TIME (hours)
PBS
Fab
anti-C5 mAb

FIG.7A

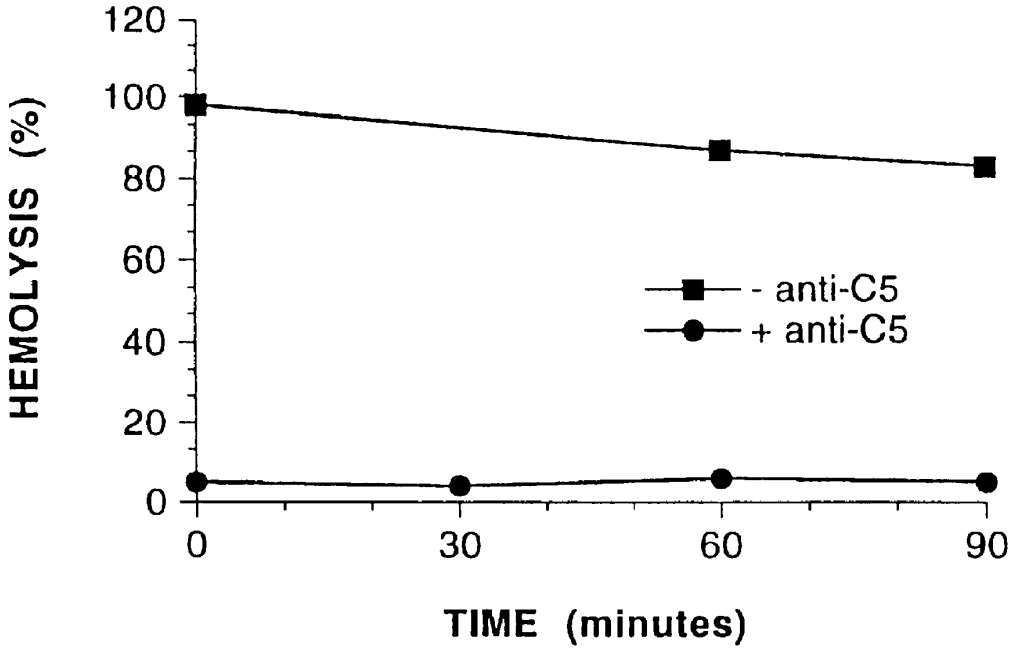

HEMOLYSIS (%)
120
100
80
60
40
20
0
0
30
60
90
TIME (minutes)
- anti-C5
+ anti-C5

FIG.7B

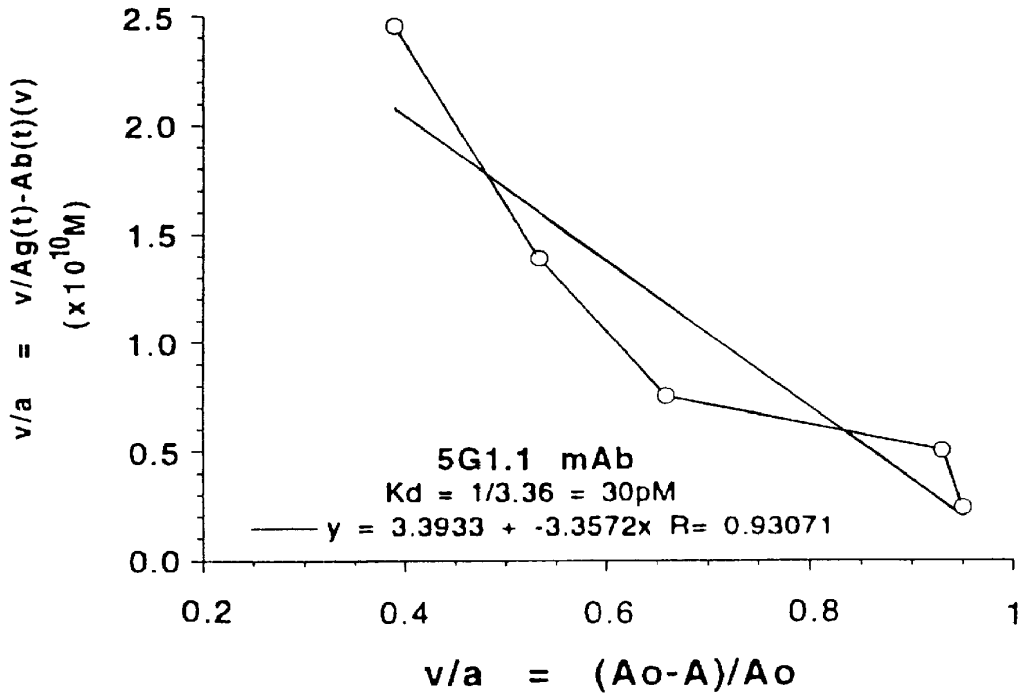

v/a = v/Ag(t)-Ab(t)(v)
(x10^10M)
2.5
2.0
1.5
1.0
0.5
0.0
0.2
0.4
0.6
0.8
1
5G1.1 mAb
Kd = 1/3.36 = 30pM
y = 3.3933 + -3.3572x R= 0.93071
v/a = (Ao-A)/Ao

FIG.8

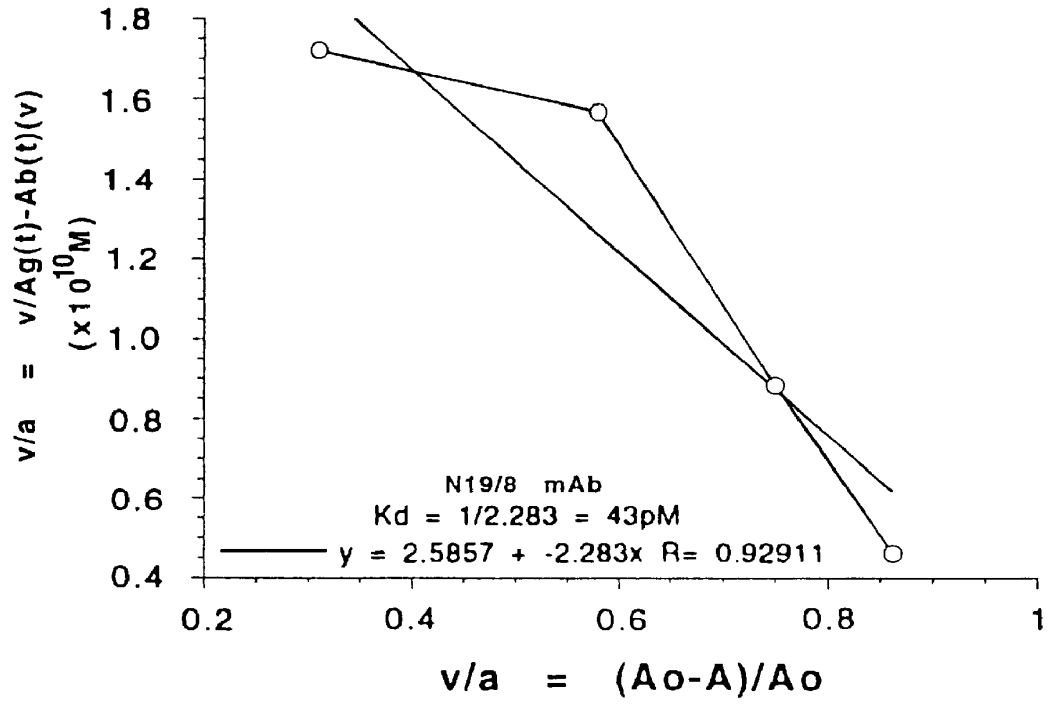

1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
v/a = v/Ag(t)-Ab(t)(v)
(x10^10 M)
N19/8 mAb
Kd = 1/2.283 = 43pM
y = 2.5857 + -2.283x R= 0.92911
0.2
0.4
0.6
0.8
1
v/a = (Ao-A)/Ao

FIG.9

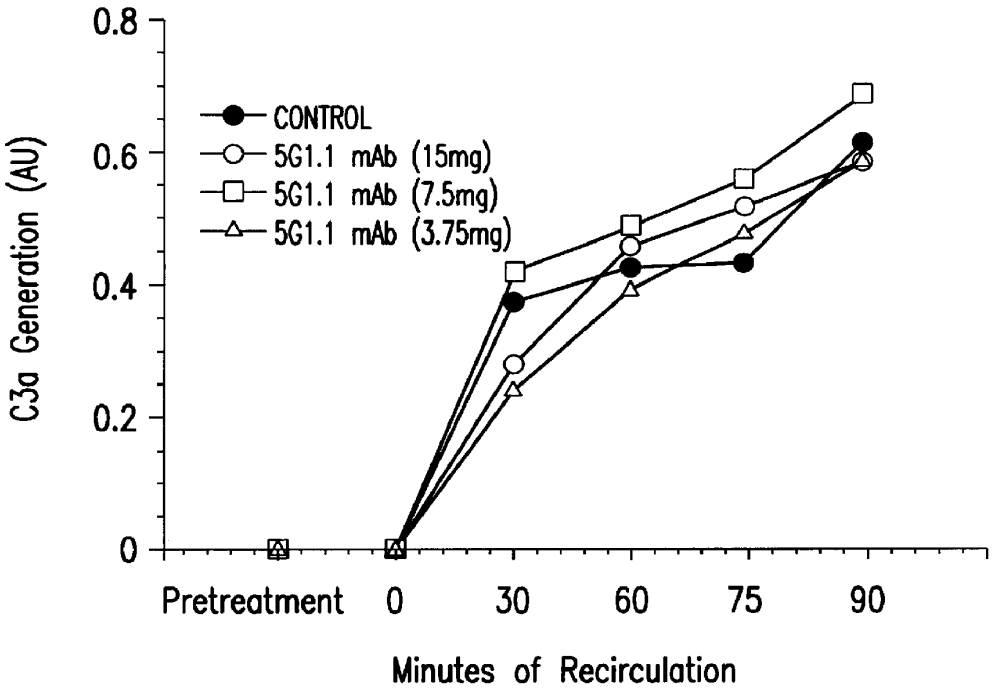

0.8
0.6
0.4
0.2
0
C3a Generation (AU)
CONTROL
5G1.1 mAb (15mg)
5G1.1 mAb (7.5mg)
5G1.1 mAb (3.75mg)
Pretreatment
0
30
60
75
90
Minutes of Recirculation

FIG.10

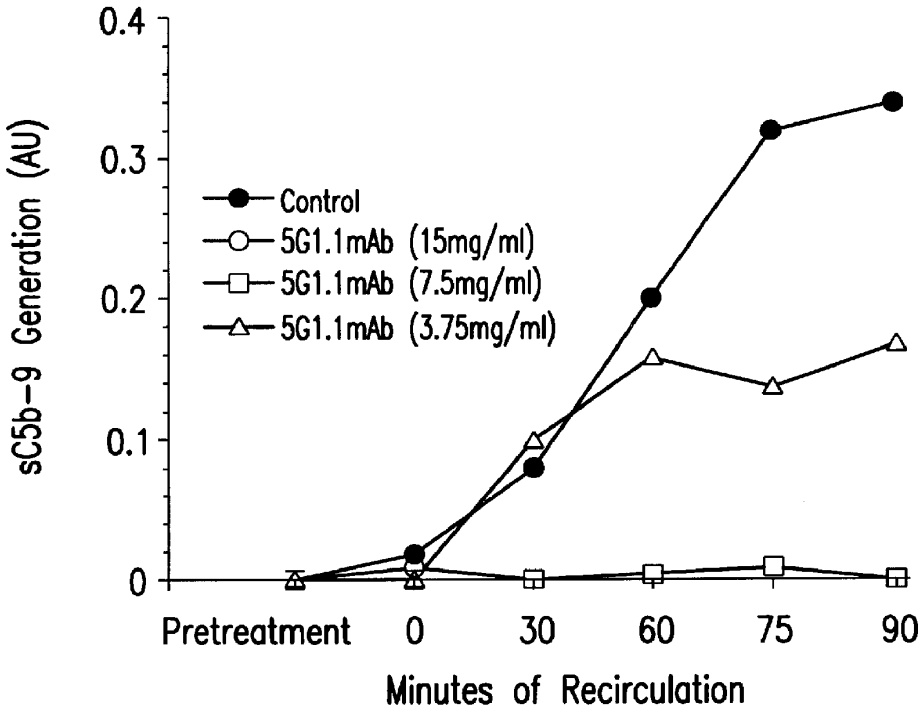

0.4
0.3
0.2
0.1
0
sC5b-9 Generation (AU)
Control
5G1.1mAb (15mg/ml)
5G1.1mAb (7.5mg/ml)
5G1.1mAb (3.75mg/ml)
Pretreatment
0
30
60
75
90
Minutes of Recirculation

FIG. 11

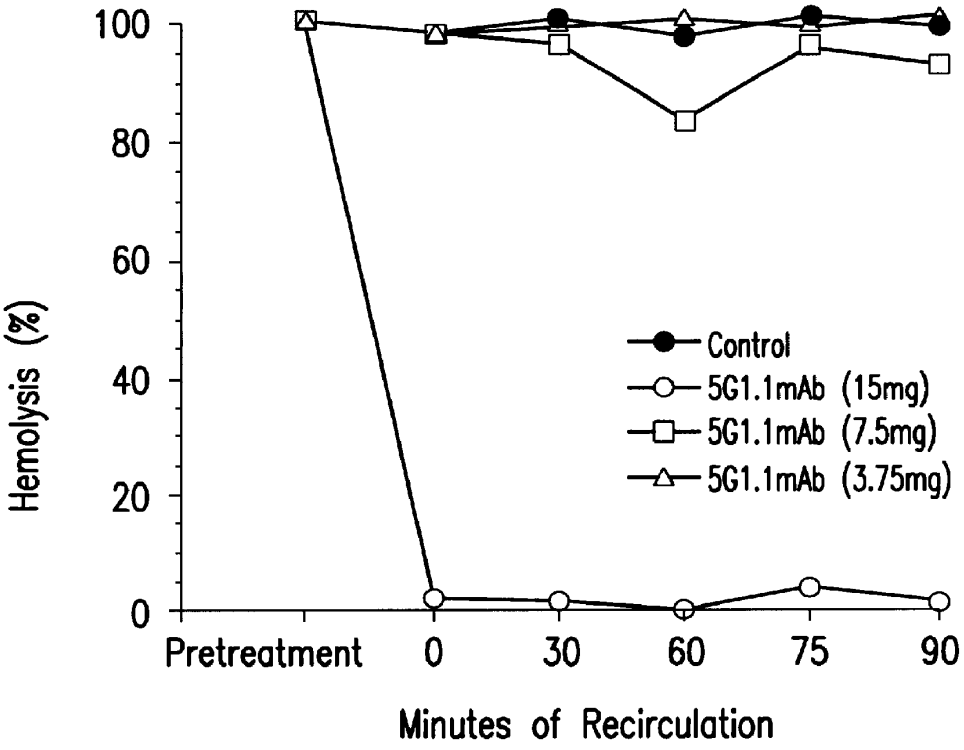

100
80
60
40
20
0
Hemolysis (%)
Pretreatment
0
30
60
75
90
Minutes of Recirculation
Control
5G1.1mAb (15mg)
5G1.1mAb (7.5mg)
5G1.1mAb (3.75mg)

FIG. 12

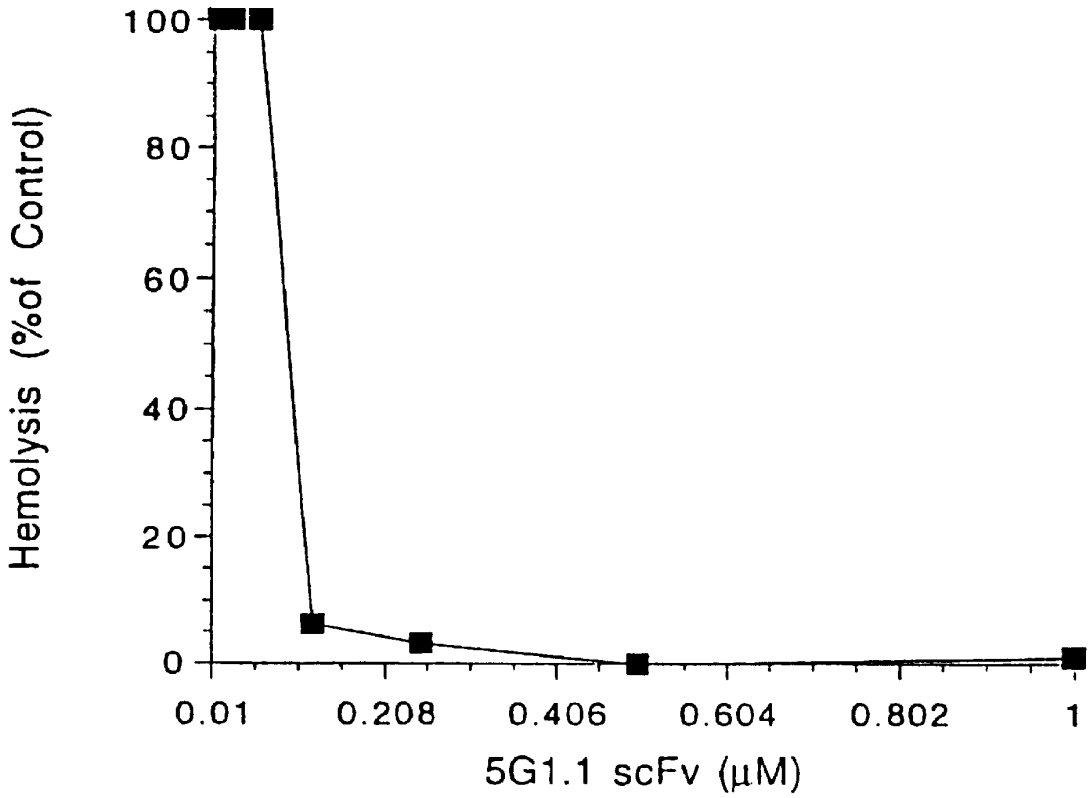

100
80
60
40
20
0
Hemolysis (%of Control)
0.01
0.208
0.406
0.604
0.802
1
5G1.1 scFv (μM)

FIG.13

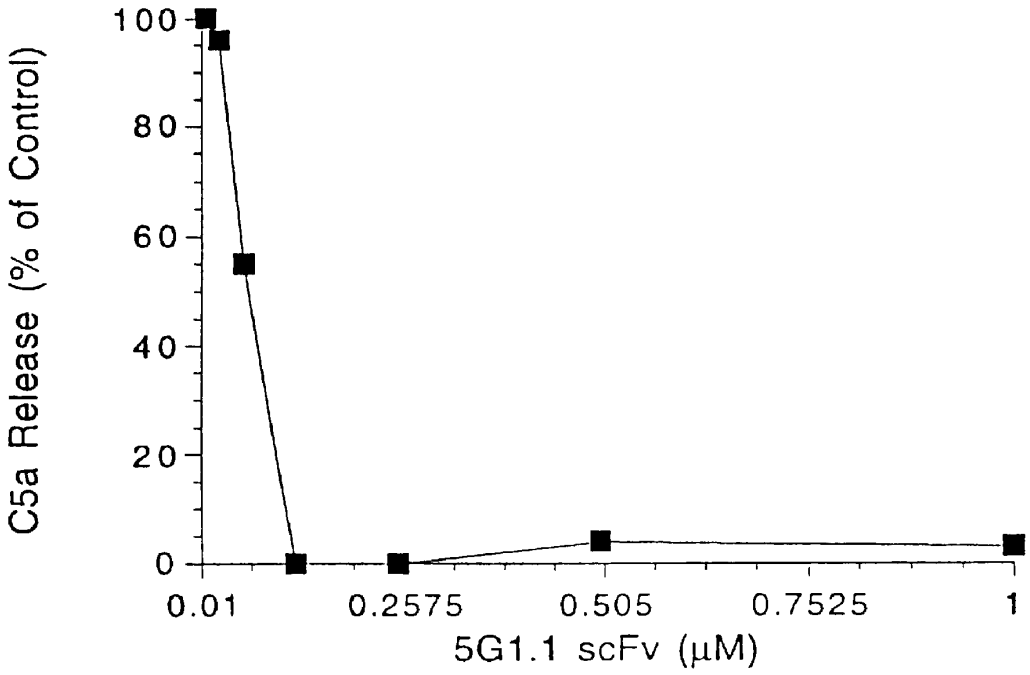

C5a Release (% of Control)
100
80
60
40
20
0
0.01
0.2575
0.505
0.7525
1
5G1.1 scFv (μM)

FIG.14

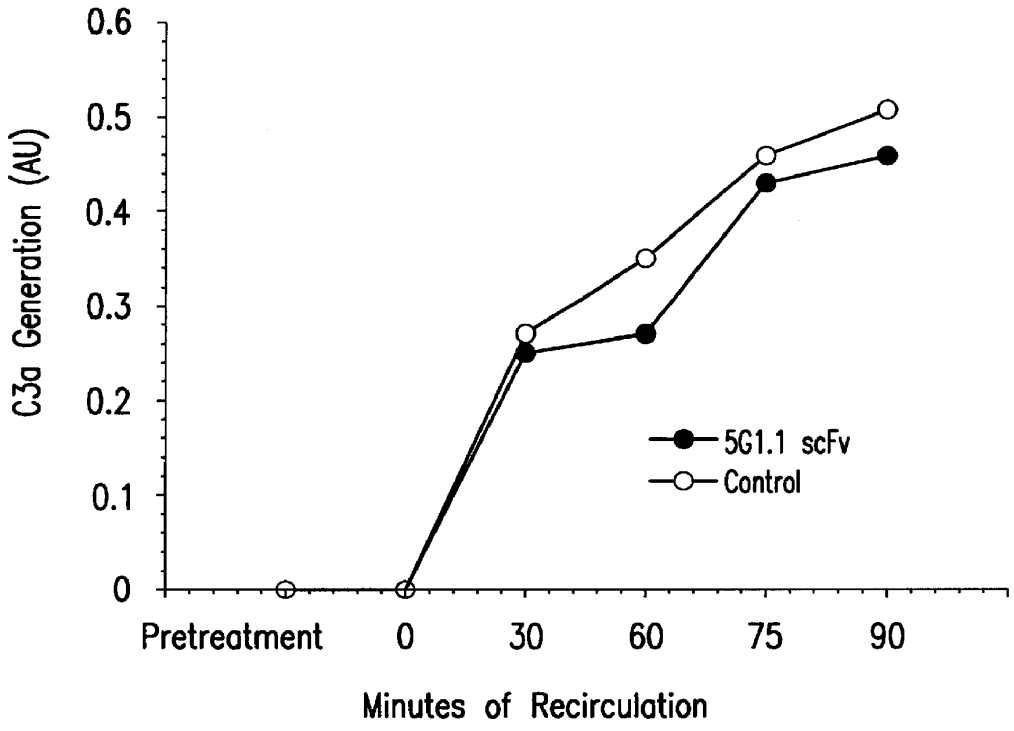

0.6
0.5
0.4
0.3
0.2
0.1
0
C3a Generation (AU)
5G1.1 scFv
Control
Pretreatment
0
30
60
75
90
Minutes of Recirculation

FIG. 15

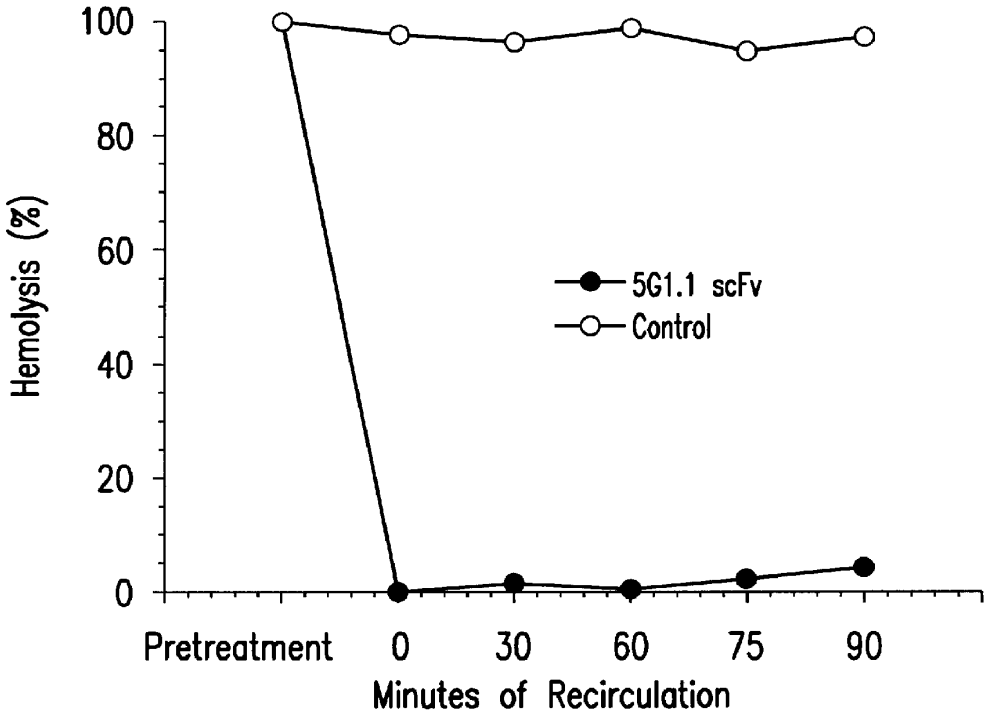

100
80
60
40
20
0
Hemolysis (%)
5G1.1 scFv
Control
Pretreatment
0
30
60
75
90
Minutes of Recirculation

FIG. 16

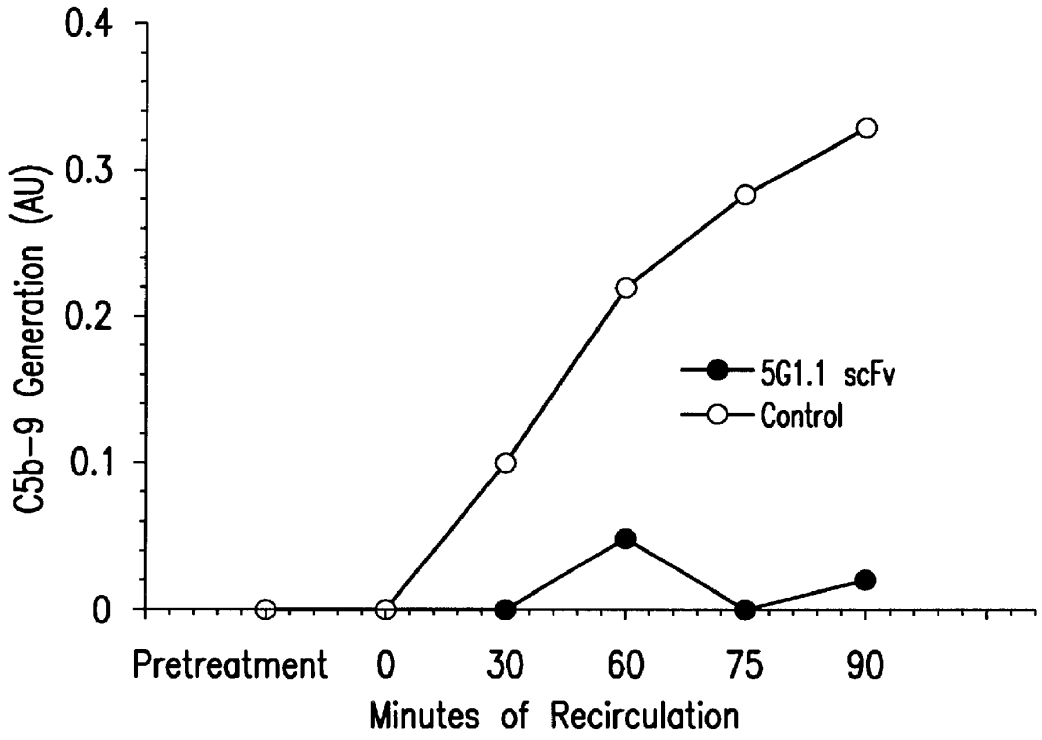

0.4
0.3
0.2
0.1
0
C5b-9 Generation (AU)
5G1.1 scFv
Control
Pretreatment
0
30
60
75
90
Minutes of Recirculation

FIG. 17

```
1                                                                  10                                                                  20
GAC ATC CAG ATG ACT CAG TCT CCA GCT TCA CTG TCT GCA TCT GTG GGA GAA ACT GTC ACC
 D   I   Q   M   T   Q   S   P   A   S   L   S   A   S   V   G   E   T   V   T

                    24                                  30                  34                                  40
ATC ACA TGT GGA GCA AGT GAG AAT ATT TAC GGT GCT TTA AAT TGG TAT CAG CGG AAA CAG
 I   T   C   G   A   S   E   N   I   Y   G   A   L   N   W   Y   Q   R   K   Q
                                CDR-L1

                                    50                                  56                                  60
GGA AAA TCT CCT CAG CTC CTG ATC TAT GGT GCA ACC AAC TTG GCA GAT GGC ATG TCA TCG
 G   K   S   P   Q   L   L   I   Y   G   A   T   N   L   A   D   G   M   S   S
                                CDR-L2

                                    70                                  80
AGG TTC AGT GGC AGT GGA TCT GGT AGA CAG TAT TAT CTC AAG ATC AGT AGC CTG CAT CCT
 R   F   S   G   S   G   S   G   R   Q   Y   Y   L   K   I   S   S   L   H   P

                    89                                  97                  100
GAC GAT GTT GCA ACG TAT TAC TGT CAA AAT GTG TTA AAT ACT CCT CTC ACG TTC GGT GCT
 D   D   V   A   T   Y   Y   C   Q   N   V   L   N   T   P   L   T   F   G   A
                                CDR-L3

GGG ACC AAG TTG GAG CTG AAA
 G   T   K   L   E   L   K
```

FIG.18

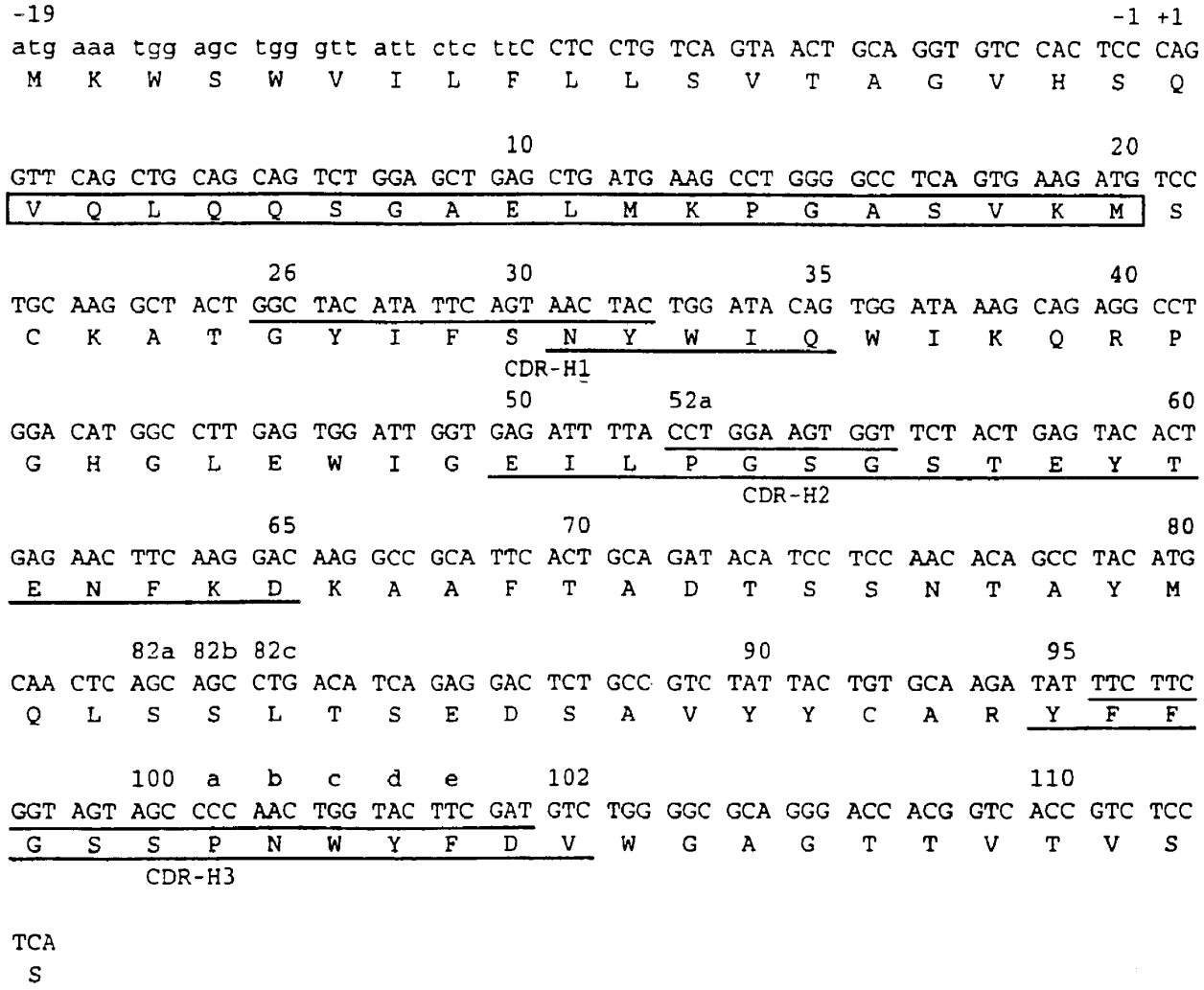

-19 -1 +1
atg aaa tgg agc tgg gtt att ctc ttC CTC CTG TCA GTA ACT GCA GGT GTC CAC TCC CAG
M K W S W V I L F L L S V T A G V H S Q
10 20
GTT CAG CTG CAG CAG TCT GGA GCT GAG CTG ATG AAG CCT GGG GCC TCA GTG AAG ATG TCC
V Q L Q Q S G A E L M K P G A S V K M S
26 30 35 40
TGC AAG GCT ACT GGC TAC ATA TTC AGT AAC TAC TGG ATA CAG TGG ATA AAG CAG AGG CCT
C K A T G Y I F S N Y W I Q W I K Q R P
CDR-H1
50 52a 60
GGA CAT GGC CTT GAG TGG ATT GGT GAG ATT TTA CCT GGA AGT GGT TCT ACT GAG TAC ACT
G H G L E W I G E I L P G S G S T E Y T
CDR-H2
65 70 80
GAG AAC TTC AAG GAC AAG GCC GCA TTC ACT GCA GAT ACA TCC TCC AAC ACA GCC TAC ATG
E N F K D K A A F T A D T S S N T A Y M
82a 82b 82c 90 95
CAA CTC AGC AGC CTG ACA TCA GAG GAC TCT GCC GTC TAT TAC TGT GCA AGA TAT TTC TTC
Q L S S L T S E D S A V Y Y C A R Y F F
100 a b c d e 102 110
GGT AGT AGC CCC AAC TGG TAC TTC GAT GTC TGG GGC GCA GGG ACC ACG GTC ACC GTC TCC
G S S P N W Y F D V W G A G T T V T V S
CDR-H3
TCA
S

FIG.19

C5-SPECIFIC ANTIBODIES FOR THE TREATMENT OF INFLAMMATORY DISEASES

This is a continuation in part of International application Ser. No. PCT/US95/05688, filed May 1, 1995, which is a continuation in part of U.S. application Ser. No. 08/236,208, filed May 2, 1994, which issued as U.S. Pat. No. 6,074,642 on Jun. 13, 2000. International application Serial No. PCT/US95/05688 was published in English under PCT Article 21(2) on Nov. 9, 1995 as WO 95/29697.

FIELD OF THE INVENTION

The present invention relates to the treatment of glomerulonephritis (GN) and other inflammatory diseases, and more generally to therapeutic treatments involving the pharmacologic inhibition of a patient's complement system. In particular, the invention relates to the use of antibodies specific to human complement component C5 to accomplish such therapeutic treatment. The invention also relates to compositions comprising native monoclonal antibodies (mAbs) specific to human complement component C5 that block complement hemolytic activity and C5a generation at concentrations that substantially reach the theoretical one to two stoichiometric limit of antibody to antigen that can be achieved by a bivalent antibody. The invention further provides recombinant mabs that are derivatives (including monovalent derivatives) of these native mAbs that provide substantially the same blocking activities as the native mabs.

BACKGROUND OF THE INVENTION

I. Immune Complex Mediated Disease

The formation of immune complexes is the typical consequence of the interaction of antigens with specific antibodies. The inflammatory response that ensues when such complexes accumulate in a limited area is an important element of normal host defenses, leading to immune complex clearance and antigen destruction by phagocytic cells. In contrast, immune complex diseases are reflections of excess complex formation or retarded clearance, usually under conditions of exceptional antigen challenge or immunologic dysregulation. Under such circumstances, immune complexes are deposited or formed at specific tissue sites and resulting inflammatory responses lead to disease states due to localized or systemic tissue damage. The kidney, and more specifically the kidney structure known as the glomerulus, is a particularly important site of immune complex deposition resulting in the development of serious disease conditions.

Human studies, and studies using animal models of human diseases, have implicated the complement system in the pathologies associated with a number of immune complex associated disorders. The activation of complement that mediates the pathology associated with these disorders may be a consequence of an autoimmune mechanism, or can be non-immunologic in origin.

The hypersensitivity response that occurs when antibodies bind to antigens either in tissues or in the circulation results from the activation of complement and the release of molecules that mediate inflammation. This process is classified as either being mediated by the binding of antibody to fixed tissue or cell bound antigens (Type II hypersensitivity) or to circulating antigens, resulting in the formation of circulating immune complexes and their subsequent pathogenic deposition in tissues (Type III hypersensitivity).

Type II hypersensitivity is mediated through the activation of complement following the binding of antibodies to fixed tissue antigens. The inflammatory response that ensues results from the activation of the proinflammatory and lytic components of the complement system and the subsequent recruitment of stimulated leukocytes to the sites of immune complex formation. The increased vascular permeability that results from the anaphylatoxic activities of C3a and C5a further enhances immune complex deposition and leukocyte recruitment.

The cross-linking of antibody bound cells or tissues to effector cells such as neutrophils, platelets, NK cells, and monocytes via their Fc receptors also plays a proinflammatory role. Such cross-linking activates effector cells, stimulating the release of oxygen radicals, prostaglandins, and leukotrienes, which release is further potentiated by the actions of activated complement components.

Examples of Type II hypersensitivity-mediated conditions include hyperacute rejection of transplanted organs, autoimmune hemolytic and thrombocytopenic states, Goodpasture's syndrome (and associated glomerulonephritis and pulmonary hemorrhage), myasthenia gravis, pathologic sequellae associated with insulin-dependent diabetes melitus, and pemphigus vulgaris.

Type III hypersensitivity reactions involving circulating antigens can also result in the development of numerous pathologic conditions. These include glomerulonephritis (discussed in detail below), vasculitis (a potentially life-threatening inflammatory condition of large and/or small blood vessels), rheumatoid arthritis, dermatitis, and other disorders.

Other diseases associated with type III hypersensitivity reactions include autoimmune diseases such as systemic lupus erythematosis (SLE), many infectious diseases, neoplastic diseases, and a wide variety of other conditions (Dixon, et al. *Immune Complex Injury*, in Samter, (ed.) Immunological Diseases, 4th ed. Little Brown & Co. Boston, 1987).

II. Glomerulonephritis

The glomerulus is a key structural and functional element of the kidney. Each glomerulus is found as part of a larger structure that serves as the main functional unit of the kidney and is called a nephron. About a million nephrons are found in each kidney. Each glomerulus is a network of up to fifty parallel capillaries encased in a structure known as Bowman's capsule. The area inside Bowman's capsule that is not taken up by the glomerular capillaries is known as Bowman's space. The glomerulus functions as a filter, separating water and certain solutes from the proteins and cells of the blood into Bowman's space for further processing in the convoluted tubules, loop of Henle, and collecting duct of the nephron.

Glomerulonephritis (GN) is a disease of the kidney characterized by inflammation and resulting enlargement of the glomeruli that is typically due to immune complex formation. The accumulation of immune complexes in the glomeruli results in inflammatory responses, involving inter alia hypercellularity, that can cause total or partial blockage of the glomerulus through, among other factors, narrowing of capillary lumens. One result of this process is the inhibition of the normal filtration function of the glomerulus. Blockage may occur in large numbers of glomeruli, directly compromising kidney function and often causing the abnormal deposition of proteins in the walls of the capillaries making up the glomerulus. Such deposition can, in turn, cause damage to glomerular basement membranes. Those glomeruli that are not blocked develop increased permeability, allowing large amounts of protein to pass into the urine, a condition referred to as proteinuria.

In many cases of severe GN, pathological structures called crescents are formed within the Bowman's space, further impeding glomerular filtration. These structures can only be seen by microscopic examination of tissue samples obtained by biopsy or necropsy, and are thus not always observed in those patients in which they occur. Crescents are a manifestation of hypercellularity and are thought to arise from the extensive abnormal proliferation of parietal epithelial cells, the cells that form the inner lining of the Bowman's capsule. Clinical research has shown that there is a rough correlation between the percentage of glomeruli with crescents and the clinical severity of the disease, and thus the patient's prognosis. When present in large numbers, crescents are a poor prognostic sign.

Symptoms of GN include: proteinuria; reduced glomerular filtration rate (GFR); serum electrolyte changes including azotemia (uremia, excessive blood urea nitrogen—BUN) and salt retention, leading to water retention resulting in hypertension and edema; hematuria and abnormal urinary sediments including red cell casts; hypoalbuminemia; hyperlipidemia; and lipiduria.

In 1990, over 210,000 patients in the United States required hemodialysis or transplantation for chronic renal failure at an annual cost in excess of 7 billion dollars, according to the United States Renal Data System (USRDS). The USRDS compiles data on kidney disease in the United States in conjunction with the National Institute of Diabetes and Digestive and Kidney Diseases, Division of Kidney, Urologic, and Hematologic Diseases, of the National Institutes of Health (NIDDKD). The USRDS estimates that the costs of treatment for renal failure are now increasing by 20 percent annually.

GN is the third leading cause of death in end-stage renal disease patients, exceeded only by diabetes and hypertension. As a result, there is a clear and long felt need in the medical community for effective treatments for this condition. Research aimed at the development of new treatments for GN is ongoing worldwide. In the United States, the NIDDKD, the National Kidney Foundation, and several other public and private organizations sponsor research in this area. The National Kidney Foundation alone supplies over two million dollars annually to fund the efforts of kidney researchers.

III. Current Treatments for GN

Corticosteroid administration, typically as high doses of "pulse" intravenous methylprednisolone or oral prednisone therapy, is currently considered the most effective pharmacologic agent available for the treatment of GN. Such steroid therapy is often administered in combination with cytotoxic general immunosuppressive agents such as azathioprine or cyclophosphamide. The overall immune suppression and resulting increased susceptibility to infection, along with other debilitating side effects associated with both steroid and cytotoxic drug administration, limit the effective use of these drugs.

Aspirin-like non-steroidal anti-inflammatory drugs (NSAIDs) have also been used to reduce the glomerular inflammation and enlargement of GN. These drugs are not routinely used for this purpose, however, probably because of their relatively weak anti-inflammatory effects and propensity to cause gastrointestinal and other side effects in many patients.

The administration of anticoagulants such as heparin or warfarin sodium, and antithrombotic agents such as cyproheptadine, dipyridamole, or sulfinpyrazone, has been used on the basis of evidence suggesting the involvement of the coagulation process in the genesis of glomerular crescents. However, objective evidence of benefit from such therapies in animals afflicted with experimentally induced crescentic GN has been inconsistent. Also, anticoagulants are dangerous drugs, as they can potentiate life-threatening bleeding episodes. They are especially hazardous in this regard in patients with advanced renal failure.

In addition to pharmacologic approaches, intensive plasma exchange (plasmapheresis) of 2 to 4 liters of plasma daily (or in some cases three times a week) can dramatically reduce high levels of circulating immune complexes when acute intervention in the inflammatory process is needed. Such treatment is expensive and requires that the patient be connected to the plasmapheresis machine for many hours each week. In addition, all procedures in which blood is removed from and returned to a patient are associated with an increased risk of infection. Nonetheless, plasma exchange is currently considered the most effective non-pharmacological treatment for removal of circulating immune complexes which can cause GN.

Circulating immune complex levels can also be decreased by eliminating or reducing the source of the antigen or antigens contained in the complexes by, for example, effective therapy of an underlying infection or change in an antibiotic. However, while such therapy is almost always a treatment of choice, great care must be taken since reduction of the antigen load alters the molar ratio of antigen to antibody involved in forming immune complexes and thus a dangerous temporary exacerbation of the inflammatory process may occur (see discussion below in Background Physiology & Pathology).

IV. Antibody Engineering

Native antibodies are multi-subunit animal protein molecules with highly specific antigen-binding properties. Animals make multiple classes of antibodies. There are five major classes (IgA, IgD, IgE, IgG and IgM) and a variety of subclasses. Native antibodies are made up of two or more heterodimeric subunits each containing one heavy (H) and one light (L) chain. The differences between antibody classes derive from their different H chains. H chains have a molecular weight of about 53 kDa, while L chains are about 23 kDa in mass.

Every individual native antibody has one type of L chain and one type of H chain, which are held together by disulfide bonds to form a heterodimeric subunit. Typically a native antibody (e.g., an IgG) has two such subunits, which are also held together by disulfide bonds. Within each chain, units of about 110 amino acid residues fold so as to form compact domains. Each domain is held together by a single intrachain disulfide bond. L chains have two domains, while H chains have four or five. Most H chains have a hinge region after the first (i.e., most amino-terminally located) two domains. The disulfide bonds linking together the heterodimeric subunits are located at the hinge regions. The hinge region is particularly sensitive to proteolytic cleavage, such proteolysis yielding two or three fragments (depending on the precise site of cleavage), a non-antigen binding fragment containing only H chain C regions (Fc) and one bivalent (Fab'2) or two monovalent (Fab) antigen binding fragments. The hinge region allows the antigen binding regions (each made up of a light chain and the first two domains of a heavy chain) to move freely relative to the rest of the native antibody, which includes the remaining heavy chain domains.

The first domain of each chain is highly variable in amino acid sequence, providing the vast spectrum of antibody binding specificities found in each individual. These are known as variable heavy (VH) and variable light (VL)

domains. The second and subsequent (if any) domains of each chain are relatively invariant in amino acid sequence. These are known as constant heavy (CH) and constant light (CL) domains.

Each variable region contains three loops of hypervariable sequence that provide a complementary structure to that of the antigen and are critical in determining the antigen binding specificity of the antibody, as they are the contact sites for binding to the antigen. These loops are known as complementarity determining regions, or CDRs. Each variable domain is made up of three CDRs embedded in four much less variable framework segments (FRs). Together, the sets of collinear CDRs and FRs are in large part responsible for determining the three dimensional conformation of the variable regions of antibody molecules.

CDRs and FRs are features that have been deduced from structural properties of antibody variable regions. Both amino acid sequence (primary structure) and three dimensional modeling (deduced secondary and tertiary structure) of antibody variable regions have been used by various researchers to define CDRs and, by default, FRs. While the positions of the CDRs are beyond question, not all workers in the art agree upon the precise locations of the boundaries of each CDR in VH or VL regions; there is no clear cut structural marker delineating CDR/FR boundaries.

Two definitions of CDR location are currently in general use in the art. These are the "sequence variability" definition of Kabat et al. ("Sequences of Proteins of Immunological Interest," 4th ed. Washington, D.C.: Public Health Service, N.I.H.) and the "structural variability" definition of Chothia and Lesk (J. Mol. Biol. 1987, 196:901). As used herein, the terms VL CDR1, VL CDR2, VL CDR3, VH CDR1, VH CDR2, and VH CDR3 refer minimally to the region of overlap between the regions designated for each CDR by each of these two definitions, and maximally to the total region spanned by the combination of the regions designated for each CDR by each of these two definitions.

One problem that antibody engineering attempts to address is the immune activity of a human patient that occurs in response to a native murine (or other non-human animal) antibody, typically a mAb, that is being administered to the patient for therapeutic purposes. This activity against murine antibodies is characterized by a human anti-mouse antibody (HAMA) response that can have deleterious effects on treatment efficacy and patient health. It has been found that almost all such human anti-non-human antibody ("HAMA type") activity is directed at the constant domains and at the FR regions of the variable domains of native non-human antibodies.

By manipulating the nucleic acid molecules encoding antibody H and L chains it is possible to incorporate non-human variable regions into antibodies otherwise made up of human constant regions. The resulting antibodies are referred to as "chimeric antibodies," and are typically less prone to eliciting HAMA type responses than are the non-human antibodies from which the variable regions are derived.

An even more effective approach to eliminating the potential of a non-human antibody to elicit a HAMA type response is to "humanize" it, i.e., to replace its non-human framework regions with human ones. One way of achieving such humanization involves the insertion of polynucleotide fragments encoding the non-human CDRs of the antibody to be humanized into a nucleic acid molecule encoding an otherwise human antibody (with human constant regions if desired) so as to replace the human CDRs and to use the resulting nucleic acid molecule to express the encoded "humanized" antibody.

Unfortunately, however, humanization of non-human antibodies has unpredictable effects on antibody antigen interactions, e.g., antigen binding properties. Some of this unpredictability stems from the properties of the CDRs. Certain CDRs may be more amenable to the construction of humanized antibodies that retain the properties of the non-human CDR donor antibody than others. While the CDRs are key to the antigen binding properties of an antibody, CDRs and FRs must interact appropriately if the antigen specificity of an antibody is to be retained following humanization. The effects of combination with particular human FRs on uncharacterized non-human CDRs cannot be reliably predicted by any known method. However, the successful humanization of an antibody provides information that, in general, facilitates the successful humanization of the CDRs of that antibody using other human or altered human FRs. In addition, approaches are available that facilitate tailoring human FRs to enhance the likelihood of successful humanization.

Other problems addressed by antibody engineering include efficient antibody production and alteration of antibody pharmacokinetics. Recombinant protein production is generally most efficiently carried out in bacterial hosts. The large size and multimeric nature of native antibodies makes their production in bacteria difficult. One approach to dealing with production problems is to use recombinant DNA methods to construct antibodies that have their H and L chains joined by a linker peptide to form a single chain (sc) antibody. As described below, there are several types of sc antibodies that can be constructed.

As is the case for humanization, the effects on antigen binding properties of constructing a particular type of sc antibody using H and L chains that have not been characterized with regard to their ability to function as part of an sc antibody cannot be reliably predicted by any known method. However, the successful construction of any one type of sc antibody from a particular native antibody provides information that, in general, facilitates the successful construction of other types of sc antibodies from that native antibody.

Single chain antibodies may include one each of only VH and VL domains, in which case they are referred to as scFv antibodies; they may include only one each of VH, VL, CH, and CL domains, in which case they are referred to as scFab antibodies; or they may contain all of the variable and constant regions of a native antibody, in which case they are referred to as full length sc antibodies.

The differing sizes of these antibodies imparts each with differing pharmacokinetic properties. In general, smaller proteins are cleared from the circulation more rapidly than larger proteins of the same general composition. Thus, full length sc antibodies and native antibodies generally have the longest duration of action, scFab antibodies have shorter durations of action, and scFv antibodies have even shorter durations of action. Of course, depending upon the illness being treated, longer or shorter acting therapeutic agents may be desired. For example, therapeutic agents for use in the prevention of immune and hemostatic disorders associated with extracorporeal circulation procedures (which are typically of brief duration) are preferably relatively short acting, while antibodies for the treatment of long term chronic conditions (such as inflammatory joint disease or GN) are preferably relatively long acting.

Detailed discussions of antibody engineering may be found in numerous recent publications including: Borrebaek, "Antibody Engineering, A Practical Guide," 1992, W.H. Freeman and Co. NY; and Borrebaek, "Antibody Engineering," 2nd ed. 1995, Oxford University Press, NY, Oxford.

SUMMARY OF THE INVENTION

In view of the foregoing, it is an object of the present invention to provide a new approach for reducing the glomerular inflammation and kidney dysfunction associated with GN.

The method of the invention involves the use of preparations containing antibodies to human complement component C5 as pharmaceutical agents. More particularly, the invention provides for the use of anti-C5 antibodies that bind to complement component C5 or active fragments thereof. Preferably, the antibodies block the generation and/or activity of complement components C5a and C5b. For most applications, the antibody is a monoclonal antibody.

In the preferred embodiments of the invention, the administration of the anti-C5 antibody preparation is started after the appearance of GN symptoms, e.g., after the appearance of proteinuria. Alternatively, the invention can be used prophylactically to treat patients who are at risk for an acute exacerbation of existing GN, e.g., patients experiencing a flare-up of symptoms of systemic lupus erythematosus or similar autoimmune diseases that have resulted in GN.

As shown in the examples presented below, anti-C5 antibodies administered subsequent to the onset of GN essentially eliminate glomerular inflammation/enlargement and reduce kidney dysfunction (see Examples 1 and 2).

Although not wishing to be bound by any particular theory of operation, it is believed that the anti-C5 antibodies have these and other therapeutic effects through their activity in blocking the generation or activity of the C5a and/or C5b active fragments of complement component C5. Through this blocking effect, the antibodies inhibit the proinflammatory (anaphylatoxic) effects of C5a and the generation of the C5b-9 membrane attack complex (MAC). Significantly, the blockage effected by the anti-C5 antibodies, since it occurs at the level of complement component C5, has the advantage of maintaining important opsonic, anti-infective, and immune complex clearance functions of the complement system mediated by, inter alia, complement component C3.

The invention additionally provides compositions comprising anti-C5 antibodies that block complement hemolytic activity and C5a generation. These antibodies are useful for the treatment of GN as well as a number of other conditions. These include treatment of immune and hemostatic dysfunctions associated with extracorporeal circulation (see copending U.S. patent application Ser. No. 08/217,391, now U.S. Pat. No. 5,853,722 which is incorporated herein by reference), treatment of inflammatory joint diseases (see copending U.S. patent application Ser. No. 08/311,489, which is incorporated herein by reference), and other complement associated conditions, particularly inflammatory diseases.

Although other antibodies can be used to treat GN in accordance with the present invention, the novel antibodies of the invention are preferred. Preferably, these novel antibodies bind to the alpha chain of C5, but do not exhibit substantial binding to the alpha chain cleavage product C5a (referred to hereinafter and in the claims as "free C5a"). Other preferred targets for antibody binding include fragments of the alpha chain of human C5 that are immunoreactive with the most preferred antibody of the invention, the 5G1.1 antibody discussed below. Such preferred targets include the 46 kDa acid hydrolysis fragment of C5 (the "5G46k" fragment), the 27 kDa tryptic digestion fragment of C5 (the "5G27k" fragment), the 325aa peptide spanning amino acid residues 725–1049 of SEQ ID NO:2 (the "5G325aa" peptide), the 200 amino acid peptide spanning amino acids residues 850 to 1049 of SEQ ID NO:2 (the "5G200aa" peptide)—as discussed below in Example 13.

The novel antibodies of the invention include antibodies that bind to an epitope within the amino acid sequence Val Ile Asp His Gln Gly Thr Lys Ser Ser Lys Cys Val Arg Gln Lys Val Glu Gly Ser Ser, (SEQ ID NO:1) hereinafter referred to as the KSSKC epitope. These novel antibodies that bind to the KSSKC epitope (SEQ ID NO:1) are hereinafter referred to as anti-KSSKC antibodies, and monoclonal antibodies binding to the KSSKC epitope are hereinafter referred to as anti-KSSKC mAbs.

The novel antibodies of the invention have many advantages over other anti-C5 antibodies, particularly with regard for their use as anti-inflammatory therapeutic agents. These include the ability to substantially block both complement hemolytic activity and the generation of the proinflammatory complement cleavage product C5a to substantially the same extent at the same concentration of antibody. Some of the preferred antibodies of the invention have the additional advantageous property of blocking the binding of C5 to C3 or C4.

Particularly preferred antibodies of the invention are monospecific native anti-KSSKC antibodies. The 5G1.1 native anti-KSSKC mAb has the distinct advantage of substantially blocking both complement hemolytic activity and the generation of C5a at a stoichiometric ratio of antibody to C5 that approaches the theoretical one to two (antibody to antigen) limit of binding that can be achieved by a bivalent antibody. This is a desirable property because it allows smaller doses of antibody to achieve therapeutic effects than would be required of otherwise similar antibodies that cannot function at such a ratio.

The invention further provides recombinant mAbs that are derivatives (including monovalent derivatives) of these native mAbs. These include anti-KSSKC recombinant mabs. Preferably the antibodies of the invention provide a level of blockade of both complement hemolytic activity and C5a generation (on a per mole of binding site basis) that is obtained when the antibody concentration is within an order of magnitude of that of the native mAbs. Particularly preferred anti-KSSKC recombinant mAbs provide a level of such blockade when the antibody concentration is no more than three fold that of the native mAbs of the invention.

The invention further provides nucleic acid sequences of polynucleotides encoding such recombinant anti-KSSKC mAbs, as well as amino acid sequences of the polypeptides encoded by these nucleic acid molecules of the invention.

The invention further provides CDR sequences that are useful in the construction of the humanized antibodies of the invention, as well as peptides and oligopeptides that are useful in the preparation and characterization of the antibodies of the invention.

Anti-C5 antibodies of the invention have activity in blocking the generation or activity of the C5a and/or C5b active fragments of complement component C5. Through this blocking effect, the antibodies inhibit the proinflammatory (anaphylatoxic) effects of C5a and the generation of the C5b-9 membrane attack complex (MAC). Significantly, the blockage effected by the anti-C5 antibodies, since it occurs at the level of complement component C5, has the advantage of maintaining important opsonic, anti-infective, and immune complex clearance functions of the complement system mediated by, inter alia, complement component C3.

The accompanying figures, which are incorporated in and constitute part of the specification, illustrate certain aspects

of the invention, and together with the description, serve to explain the principles of the invention. It is to be understood, of course, that both the figures and the description are explanatory only and are not restrictive of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. **1A**, **1B**, and **1C**—Photomicrographs of PAS stained sections of mouse kidneys. FIG. **1A**—uninduced untreated mouse. FIG. **1B**—GN-induced PBS-(control)-treated mouse. FIG. **1C**—GN-induced anti-C5 treated mouse. Magnification for each is the same, approximately 400×.

FIGS. **2A**, **2B**, and **2C**—Photomicrographs of immunofluorescence stained sections of mouse kidneys. FIG. **2A**—uninduced untreated mouse. FIG. **2B**—GN-induced PBS-(control)-treated mouse. FIG. **2C**—GN-induced anti-C5 treated mouse. Magnification for each is the same, approximately 200×.

FIG. **3**—Results of hemolytic (cell lysis) assays of serum from GN-induced animals treated with either anti-C5 antibodies in PBS ("Anti-C5") or PBS alone ("PBS control"). Also shown are the results of assays performed with normal serum.

FIG. **4**—Results of soluble C5b-9 ("sC5b-9") assays. "ND" indicates not determined.

FIGS. **5A**, **5B**, and **5C**—Immunofluorescence photomicrographs of kidney sections stained for mouse C3. FIG. **5A**—uninduced untreated mouse. FIG. **5B**—GN-induced PBS-(control)-treated mouse. FIG. **5C**—GN-induced anti-C5 treated mouse. Magnification for each is the same, approximately 400×.

FIG. **6**—Results of C3a assays of samples of circulating human blood. "ND" indicates not determined.

FIGS. **7A** and **7B**—Pharmacokinetic analyses of the reduction of the cell lysis ability of mouse (FIG. **7A**) or human (FIG. **7B**) blood after treatment with anti-C5 antibodies.

The immunofluorescent staining of FIGS. **2** and **5** is confined to the glomerular capillary network (tuft) and thus the enlargement of the glomerulus seen in FIG. **1B** is not visible in FIGS. **2B** and **5B**.

FIG. **8**—Scatchard analysis of native 5G1.1 binding to C5.

FIG. **9**—Scatchard analysis of native N19/8 binding to C5.

FIG. **10**—C3a generation in samples of circulating human blood in the presence of native 5G1.1.

FIG. **11**—sC5b-9 generation in samples of circulating human blood in the presence of native 5G1.1.

FIG. **12**—Serum hemolytic activity of samples of circulating human blood in the presence of native 5G1.1.

FIG. **13**—Serum hemolytic activity in the presence of m5G1.1 scFv.

FIG. **14**—C5a generation in the presence of m5G1.1 scFv.

FIG. **15**—C3a generation in samples of circulating human blood in the presence of m5G1.1 scFv.

FIG. **16**—Serum hemolytic activity of samples of circulating human blood in the presence of 5G1.1 scFv.

FIG. **17**—sC5b-9 generation in samples of circulating human blood in the presence of m5G1.1 scFv.

FIG. **18**—The light chain variable region of the antibody 5G1.1. Sequence derived from the 5' oligonucleotide primer used for PCR amplification of the variable region is shown in lower case. Amino acids are number according to Kabat et al., supra. Boxed amino acids correspond to peptide sequences obtained from the mature 5G1.1 light chain or from an endoproteinase Lys C peptide of 5G1.1. The complementarity determining region (CDR) residues according to the sequence variability definition and the structural variability definition are underlined and overlined, respectively.

FIG. **19**—The heavy chain variable region of the antibody 5G1.1. Sequence derived from the 5' oligonucleotide primer used for PCR amplification of the variable region is shown in lower case. Amino acids are numbered using the scheme of Kabat et al. supra with +1 denoting the first amino acid of the processed mature variable region. Boxed amino acids correspond to peptide sequence obtained from the 5G1.1 heavy chain after treatment with pyroglutamate aminopeptidase. The complementarity determining region (CDR) residues according to the sequence variability definition or according to the structural variability definition are underlined and overlined, respectively.

BACKGROUND PHYSIOLOGY & PATHOLOGY

The discussion in this section is not limited to subject matter that qualifies as "prior art" against the present invention. Therefore, no admission of such prior art status shall be implied or inferred by reason of inclusion of particular subject matter in this discussion, and no declaration against the present inventors' interests shall be implied by reason of such inclusion.

I. Introduction

As described above, the present invention relates to therapeutic treatments for GN and other immune complex mediated diseases, as well as to the treatment of other complement mediated diseases and to the inhibition of complement component C5. To provide background for the description of the preferred embodiments and the examples presented below, we turn first to general discussions of the complement arm of the immune system, the pathophysiologic features of GN, and previous studies of the role of complement in GN pathogenesis.

General discussions of the complement system and GN can be found in, for example, Glassock and Brenner, 1994; Couser, 1993; Couser, 1992; Couser, et al, 1992; Rich, 1992; Glassock and Brenner, 1987; Robbins and Cotran, 1979; and Guyton, 1971.

II. The Complement System

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

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

The classical complement pathway is typically initiated by antibody recognition of and binding to an antigenic site on a target cell. The alternative pathway is usually antibody

independent, and can be initiated by certain molecules on pathogen surfaces. Both pathways converge at the point where complement component C3 is cleaved by an active protease (which is different in each pathway) to yield C3a and C3b. Other pathways activating complement attack can act later in the sequence of events leading to various aspects of complement function.

C3a is an anaphylatoxin (see discussion below). C3b binds to bacterial and other cells, as well as to certain viruses and immune complexes, and tags them for removal from the circulation. (C3b in this role is known as opsonin.) The opsonic function of C3b is considered to be the most important anti-infective action of the complement system. Patients with genetic lesions that block C3b function are prone to infection by a broad variety of pathogenic organisms, while patients with lesions later in the complement cascade sequence, i.e., patients with lesions that block C5 functions, are found to be more prone only to Neisseria infection, and then only somewhat more prone (Fearon, in *Intensive Review of Internal Medicine,* 2nd Ed. Fanta and Minaker, eds. Brigham and Women's and Beth Israel Hospitals, 1983).

C3b also forms a complex with other components unique to each pathway to form classical or alternative C5 convertase, which cleaves C5 into C5a and C5b. C3 is thus regarded as the central protein in the complement reaction sequence since it is essential to both the alternative and classical pathways (Wurzner, et al., *Complement Inflamm.* 8:328–340, 1991). This property of C3b is regulated by the serum protease Factor I, which acts on C3b to produce iC3b. While still functional as opsonin, iC3b cannot form an active C5 convertase.

C5 is a 190 kDa beta globulin found in normal serum at approximately 75 μg/ml (0.4 μM). C5 is glycosylated, with about 1.5–3 percent of its mass attributed to carbohydrate. Mature C5 is a heterodimer of a 999 amino acid 115 kDa alpha chain that is disulfide linked to a 656 amino acid 75 kDa beta chain. C5 is synthesized as a single chain precursor protein product of a single copy gene (Haviland et al. J. Immunol. 1991, 146:362–368). The cDNA sequence of the transcript of this gene predicts a secreted pro-C5 precursor of 1659 amino acids along with an 18 amino acid leader sequence (SEQ ID NO:2).

The pro-C5 precursor is cleaved after amino acid 655 and 659, to yield the beta chain as an amino terminal fragment (amino acid residues +1 to 655 of SEQ ID NO:2) and the alpha chain as a carboxyl terminal fragment (amino acid residues 660 to 1658 of SEQ ID NO:2), with four amino acids (amino acid residues 656–659 of SEQ ID NO:2) deleted between the two.

C5a is cleaved from the alpha chain of C5 by either alternative or classical C5 convertase as an amino terminal fragment comprising the first 74 amino acids of the alpha chain (i.e., amino acid residues 660–733 of SEQ ID NO:2). Approximately 20 percent of the 11 kDa mass of C5a is attributed to carbohydrate. The cleavage site for convertase action is at or immediately adjacent to amino acid residue 733 of SEQ ID NO:2. A compound that would bind at or adjacent to this cleavage site would have the potential to block access of the C5 convertase enzymes to the cleavage site and thereby act as a complement inhibitor.

C5 can also be activated by means other than C5 convertase activity. Limited trypsin digestion (Minta and Man, J. Immunol. 1977, 119:1597–1602; Wetsel and Kolb, J. Immunol. 1982, 128:2209–2216) and acid treatment (Yammamoto and Gewurz, J. Immunol. 1978, 120:2008; Damerau et al., Molec. Immunol. 1989, 26:1133–1142) can also cleave C5 and produce active C5b.

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

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

III. Pathophysiology of GN

Although GN may accompany an extraordinary range of pathologic processes, in general it is encountered most commonly in the course of infectious diseases, in autoimmunity, and as a consequence of therapy for some other disease process. The causative mechanism for GN is typically the deposit of circulating immune complexes in the kidney. Factors involved in the pathogenesis of GN include the specific antigen and antibody involved and the inflammatory processes that occur as a consequence of immune complex deposition.

Antigens Involved in the Formation of Immune Complexes that Cause GN

Antigens involved in the development of GN can be broadly classified as endogenous, infectious, and iatrogenic (those encountered as a consequence of medical practice). In many cases the specific antigen is unknown, although the general class can usually be identified.

The best known example of the formation of endogenous immune complexes is the DNA anti-DNA complexes produced in connection with systemic lupus erythematosus (lupus, SLE). Other important sources of endogenous antigens include malignancies in which immune complex formation may contribute to the development of paraneoplastic syndromes.

Infections with organisms of many types, particularly chronic infections, are also associated with the development of immune complexes that can cause GN. Bacterial and fungal infections that can produce such complexes include infection with certain strains of streptococci, Pseudomonas, disseminated gonococcal infection, lepromatous leprosy, subacute bacterial endocarditis, bronchopulmonary aspergillosis, secondary syphilis, and chronic infections in patients with cystic fibrosis.

Viral diseases in which immune complex deposition may be a prominent feature include hepatitis B infection, dengue, infectious mononucleosis, and subacute sclerosing panencephalitis. GN is also a prominent feature of many parasitic infestations such as the GN seen in children with quartan malaria, as well as toxoplasmosis, trypanosomiasis, and schistosomiasis.

Iatrogenic antigens constitute a special class of exogenous antigens. These include those responsible for the prototype immune complex disease, serum sickness, which follows

formation of immune complexes between heterologous serum constituents and autologous antibodies. Serum sickness was regularly seen earlier in this century when infectious diseases were frequently treated with heterologous antisera.

An iatrogenic disease essentially indistinguishable from classic serum sickness can occur as a consequence of high-dose antibiotic therapy. The serum sickness-like manifestations of immune responses to these drugs include GN and reflect the fact that certain drugs, particularly the β-lactam and sulfonamide antibiotics, are effective haptens that are capable of inducing antibody responses upon spontaneous conjugation to autologous proteins.

Factors Affecting Immune Complex Formation and Deposition

Features of both antigen and antibody determine the likelihood of pathologic immune complex formation and subsequent deposition in the kidney. Chief among these are the absolute concentrations of the reactants and their relative molar ratios.

Most antigens display multiple epitopes and typically stimulate a polyclonal antibody response. All naturally occurring antibody molecules are at least bivalent. These properties allow for the formation of an extensive antigen-antibody lattice, the size of which is determined largely by the affinity of the antibodies and the molar ratio of antigen to antibody.

In general, antibody responses begin under conditions in which antigen is present in excess to antibody, and this relative ratio changes as the antibody response increases in magnitude. Complexes formed initially are usually small and exhibit little or no pathogenic activity. In contrast, very large complexes are often formed as the amount of antigen becomes limiting, late in the course of an antibody response under conditions of antibody excess. Because these very large complexes are readily cleared by the reticuloendothelial system in the liver, they are also relatively nonpathogenic.

The formation of immune complexes that can cause GN is believed to occur during conditions of slight antigen excess or near the point of antibody-antigen equivalence, where lattice formation is maximal and lattice size is large, but not very large.

Several factors influence the speed and location of immune complex precipitation. Interactions between Fc regions of antibody molecules promote rapid precipitation of immune complexes. The role of Fc-Fc interactions in immune complex precipitation is illustrated by studies of the properties of F(ab')2 antibody fragments, which do not contain Fc regions. Although the valence of F(ab')2 fragments does not differ from that of most whole immunoglobulins, F(ab')2 antibody fragments form lattices more slowly.

Antigen charge plays a role in determining the tissue localization of sites of deposition of immune complex precipitates. Complexes with a substantial positive charge are preferentially attracted to the strong negative charge of basement membranes, particularly in the renal glomerulus.

Localized presence of antigen may largely account for certain cases of organ specific immune complex deposition. Diseases such as Goodpasture's syndrome (a rare form of GN) are typically not classified as immune complex diseases because the complexes are formed in situ in the kidney rather than being preformed in the circulation and then deposited. Once the immune complexes are formed, the subsequent inflammatory process is believed to be essentially the same as that seen following deposition of preformed complexes. However, the different mode of deposition distinguishes this syndrome from typical GN caused by circulating immune complexes.

Features of blood flow and vascular structure are also important in determining the localization of immune complex deposits. Chief among these is capillary permeability. Because their capillary endothelium is fenestrated, renal glomeruli are preferential sites for the deposition of immune complexes. Hemodynamic variables enhancing immune complex localization include turbulence of flow and increased blood pressure, both of which are present in the renal glomeruli.

Complement and Complement Receptors as Reaulators of Immune Complex Deposition

In addition to their proinflammatory functions, complement components can also inhibit immune complex deposition and resolubilize immune complex precipitates from sites of deposition. In addition, it is known that erythrocyte receptors for C3b, e.g., CR1, are important for reticuloendothelial clearance of opsonized circulating immune complexes.

Analysis of the clinical pattern of immune complex disease in patients with deficiencies of particular complement components provides information regarding the normal role of these components in the prevention of complex deposition. The incidence of immune complex disease in patients with deficiencies of Clq, Clr, Cls, C4, C2, or C3 varies from 60 to 90 percent, with the majority of these patients exhibiting a lupus-like syndrome. Immune complex disease is rarely associated with deficiencies of late-acting or alternative pathway components.

The binding of complement components to immune complexes prevents the formation of large antigen-antibody lattices and inhibits immune precipitation. This process requires activation via the classical pathway; serum that is deficient for Clq, C4, or C2 does not effectively inhibit lattice formation and complex precipitation. Classical pathway dependence may reflect the initial binding of Cl components, impeding the Fc-Fc interactions between IgG molecules that contribute to immune precipitation. This is followed by covalent binding of C3b to the complexes, which further inhibits immune precipitation and leads to solubilization of previously deposited complexes.

The solubilization process also depends upon activation of components of the alternative pathway. Consequently, by promoting clearance of immune complexes and inhibiting their deposition at sites of inflammation, complement components and their receptors serve as negative regulators of immune complex diseases that may retard disease development.

It should be noted that the present invention involves blocking the activities of complement component C5. The targeting of this component does not alter the functions of the early complement components, and thus does not compromise the negative regulatory effects on immune complex deposition of those early components.

Immune Complex-Mediated Inflammation

Basophils are important in the initiation of immune complex-mediated inflammatory responses, as capillary permeability is markedly increased by the action of vasoactive amines such as histamine and platelet-activating factor, which are released by these cells. Vascular permeability is also promoted by aggregation of platelets at sites of an inflammatory lesion, with the release of platelet-activating factor and the formation of microthrombi.

Basophil degranulation may reflect the effects of IgE antibodies, as well as the elaboration of the anaphylatoxin components of complement, C3a and C5a.

In addition to basophils and platelets, the primary cellular effectors of immune complex-mediated inflammation are polymorphonuclear leukocytes, monocytes, and macrophages.

IV. Previous Studies of the Role of Complement in GN Pathoaenesis

Extensive work has been performed in an attempt to understand the possible role of complement in the development of GN. This work has included studies of GN using a number of animal models by, among others, Unanue, et al., (1964); Cochrane, et al., (1965); Kniker, et al., (1965); Salant, et al., (1980); Groggel, et al., (1983); Falk and Jennette (1986); Jennette, et al., (1987); Passwell, et al., (1988); Schrijver, et al., (1988); Baker, et al., (1989); Schrijver, et al., (1990); Couser, et al., (1991); and Couser, et al., (1992).

These studies have shown that complement plays a role in GN pathogenesis. However, they have not established specific unequivocal roles for the various complement components. In particular, the relative roles of C3 and other anaphylatoxins compared to the roles of the terminal complement components in GN pathogenesis have not been unequivocally established. Also, some researchers have reported that complement depletion does not diminish glomerular injury. See Kniker, et al., (1965).

The foregoing work includes that of Falk and Jennette (1986), who reported results of experiments in which attempts were made to induce GN in mice having a genetic defect that resulted in a deficiency of complement component C5. The report concludes that C5 or some terminal complement component dependent on C5 plays a role in the pathogenesis of GN.

Significantly, with regard to the present invention, Falk and Jennette in no way disclose or suggest that an antibody to C5 can be used to treat GN. Indeed, it would be counterintuitive to use an antibody to treat disease which typically involves the formation and deposition of circulating antibody-antigen immune complexes. Plainly, the creation of more circulating immune complexes would seem to be the last way to go to solve a problem that can be caused by circulating immune complexes. Yet, as demonstrated by the surprising results presented below, anti-C5 antibodies have been found to effectively block GN, even though the creation of additional circulating immune complexes is inherent in their mode of action.

Baker et al. (1989), Couser et al. (1991), and Couser et al. (1992) (hereinafter referred to collectively as the "C6" work) discuss experiments in which high levels of an anti-C6 polyclonal antibody preparation were administered to rats, following which immune complexes were formed in situ in the rats' kidneys. Significantly, with regard to the present invention, the anti-C6 antibody preparation was not administered to animals with pre-existing kidney disease, i.e., it was not used as a therapeutic treatment. Moreover, the experimental protocol used in the C6 experiments did not involve circulating immune complexes, but rather involved complexes formed in situ. Accordingly, the experiments did not disclose or suggest the counterintuitive approach of the present invention wherein more circulating immune complexes are formed in the process of treating a disease state caused by circulating immune complexes.

Further, the anti-C6 antibody dosages used in the C6 work were too high for practical medical use. Specifically, these antibodies were used at a dosage of 1 gm/kg, a dosage which would correspond to 70 gm of antibody for a 70 kg (155 lb) individual. In contrast, the anti-C5 antibodies used in the practice of the present invention are used at concentrations at or below 0.1 gm/kg, i.e., a factor of at least ten times less than used in the C6 work. Indeed, as shown by the examples presented below, anti-C5 antibody dosages as low as 0.03 gm/kg, i.e., 33 times less than those used in the C6 work, have been found to achieve the therapeutic effects of the invention in treating GN. For a 70 kg individual, this antibody level corresponds to a dose of just 2.1 gms.

The novel anti-KSSKC antibodies of the invention allow the use of even lower dosage levels to treat GN and other inflammatory conditions. Based upon their level of activity in human blood, they are expected to provide complete complement inhibition at dosages below 0.005 g/kg, and to provide therapeutically effective complement inhibition at dosages below 0.003 g/kg. This 3 mg/kg dosage is one tenth the dosage discussed below in Examples 4 and 5 for the for the anti-C5 (beta chain specific) mAb N19/8. Some of the full length anti-KSSKC mabs of the invention will provide therapeutic benefits even at dosages below 0.0022 g/kg. This is the minimum dose providing complete complement inhibition as calculated from the data obtained using the anti-KSSKC 5G1.1 mAb in human blood in a CPB circuit, as discussed below in Example 9.

Accordingly, dosages of less than 0.005 g/kg are preferred, with dosages of below 0.003 g/kg being more preferred, and dosages below 0.0022 g/kg being particularly preferred. For a 70 kg individual, these antibody dosage levels correspond to a dose of less than 0.35 gms for the highest dosage of the preferred dosages, less than 0.21 gms for the more preferred dosage, and less than or equal to 0.15 gms for the most preferred dosage.

Of course, dosage levels of single chain and other recombinant mAbs of the invention must be adjusted according to their level of activity (e.g., their binding affinity, their ability to block C5 activation, and/or their ability to block complement hemolytic activity), their valency, and their molecular weight. For example, the humanized scFv anti-KSSKC mAbs of Example 11 are approximately 27 kDa, about one sixth the approximately 155 kDa mass of a native, full length IgG antibody. These antibodies completely block complement hemolytic activity and C5a generation at a ratio of 3:1, six fold greater than for native 5G1.1 (but only three fold greater when viewed in terms of numbers of antibody-antigen binding sites).

Thus, the number of molecules of each of these scFvs required to equal the effect of a single molecule of native 5G1.1 must be increased by a factor of six to adjust for the ratio at which blocking is complete. Since the mass of these molecules is approximately one sixth of the mass of native 5G1.1, dosages of the scFvs are in the same range as those for the native 5G1.1 mAb.

In addition to lowering dosage levels, the anti-C5 antibodies used in the practice of the present invention (i.e., in treating GN) achieve important therapeutic effects not achieved with the anti-C6 antibodies. Specifically, the control and test animals in the C6 work exhibited both hypercellularity and narrowing of capillary lumens. In direct contrast, no such hypercellularity or narrowing of capillary lumens was seen when diseased individuals were treated with anti-C5 antibodies (see FIG. 1).

Moreover, the anti-C5 antibodies used in the present invention achieve a reduction in glomerular enlargement, thus providing a clear demonstration of the unexpectedly powerful anti-inflammatory effects of the anti-C5 antibodies used in the practice of the invention. Nowhere in the C6 work is there any disclosure or suggestion of such a powerful anti-inflammatory effect.

V. Anti-C5 Monoclonal Antibodies that Block Complement Hemolytic Activity and Block the Generation of C5a

Anti-C5 mabs that have the desirable ability to block complement hemolytic activity and to block the generation of C5a (and are thus preferred for use in the treatment of GN and other inflammatory conditions in accordance with the present invention) have been known in the art since at least 1982 (Moongkarndi et al. Immunobiol. 1982, 162:397; Moongkarndi et al. Immunobiol. 1983, 165:323). Antibodies known in the art that are immunoreactive against C5 or C5 fragments include antibodies against the C5 beta chain (Moongkarndi et al. Immunobiol. 1982, 162:397; Moongkarndi et al. Immunobiol. 1983, 165:323; Wurzner et al. 1991, supra; Mollnes et al. Scand. J. Immunol. 1988, 28:307–312); C5a (see for example, Ames et al. J. Immunol. 1994, 152:4572–4581, U.S. Pat. No. 4,686,100, and European patent publication No. 0 411 306); and antibodies against non-human C5 (see for example, Giclas et al. J. Immunol. Meth. 1987, 105:201–209). Significantly, none of these anti-C5 mAbs has the properties of the novel anti-C5 mabs of the invention, i.e., none of them binds to the C5 alpha chain but not to the C5 cleavage product C5a, none of them has the ability to substantially block both complement hemolytic activity and the generation of C5a to substantially the same extent at the same concentration of antibody. It is noteworthy that an scFv derivative of the N19/8 antibody of Wurzner et al. 1991, supra, has been prepared, and that the N19/8 scFv has 50% less inhibitory activity towards C5a generation than the native N19/8 antibody (see Example 15). This is in contrast to the 5G1.1 scFv, which retained substantially all of its inhibitory activity towards C5a generation (see Example 12).

While not wishing to be bound by any particular theory of operation, it is believed that these distinctions are due to the specific binding characteristics of the antibodies of the invention. Accordingly, antibodies that do not bind to sites within the alpha chain of C5, and antibodies that bind to the C5 cleavage product C5a (free C5a), are believed to lack the ability to substantially block both complement hemolytic activity and the generation of C5a to substantially the same extent at the same concentration of antibody.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

As discussed above, the present invention relates to the use of anti-C5 antibodies in treating patients suffering from GN and other diseases, and to specific C5 antibodies and antibody preparations. Preferably, and when used to treat GN, the anti-C5 antibodies are used in an amount effective to substantially reduce (e.g., reduce by at least about 50%) the cell-lysing ability of complement present in the patient's blood (the "cell-lysing ability of complement present in the patient's blood" is also referred to herein as the "serum complement activity of the patient's blood"). Reduction of the cell-lysing ability of complement present in the patient's blood can be measured by methods well known in the art such as, for example, by the chicken erythrocyte hemolysis method described below under the heading "Cell Lysis Assays."

To achieve the desired reductions, the anti-C5 antibodies can be administered in a variety of unit dosage forms. The dose will vary according to the particular antibody. For example, different antibodies may have different masses and/or affinities, and thus require different dosage levels. Antibodies prepared as Fab' fragments will also require differing dosages than the equivalent intact immunoglobulins, as they are of considerably smaller mass than intact immunoglobulins, and thus require lower dosages to reach the same molar levels in the patient's blood.

The dose will also vary depending on the manner of administration, the particular symptoms of the patient being treated, the overall health, condition, size, and age of the patient, and the judgment of the prescribing physician. Dosage levels of the antibodies for human subjects are generally between about 1 mg per kg and about 100 mg per kg per patient per treatment, and preferably between about 5 mg per kg and about 50 mg per kg per patient per treatment. In terms of plasma concentrations, the antibody concentrations are preferably in the range from about 25 μg/ml to about 500 μg/ml.

Subject to the judgement of the physician, a typical therapeutic treatment includes a series of doses, which will usually be administered concurrently with the monitoring of clinical endpoints such as BUN levels, proteinuria levels, etc., with the dosage levels adjusted as needed to achieve the desired clinical outcome. Alternatively, levels of serum complement activity available in the patient's blood are monitored using the techniques set forth below under the heading "Cell Lysis Assays" to determine if additional doses or higher or lower dosage levels of antibodies are needed, with such doses being administered as required to maintain at least about a 50% reduction, and preferably about a 95% or greater reduction of serum complement activity. Other protocols can, of course, be used if desired as determined by the physician.

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

The formulations of the invention can be distributed as articles of manufacture comprising packaging material and the anti-C5 antibodies. When prepared for use in the treatment of GN, the packaging material will include a label which indicates that the formulation is for use in the treatment of kidney disease and may specifically refer to nephritis or glomerulonephritis.

The anti-C5 antibody is preferably a monoclonal antibody, although polyclonal antibodies produced and screened by conventional techniques can also be used if desired. As discussed above, the anti-C5 antibodies must be effective in reducing the cell-lysing ability of complement present in human blood. As also discussed above, this property of the antibodies can be determined by methods well known in the art such as, for example, by the chicken erythrocyte hemolysis method described below under the heading "Cell Lysis Assays".

The anti-C5 antibodies used in the practice of the invention bind to C5 or fragments thereof, e.g., C5a or C5b. Preferably, the anti-C5 antibodies are immunoreactive against epitopes on the beta chain of purified human complement component C5 and are capable of blocking the conversion of C5 into C5a and C5b by C5 convertase. This capability can be measured using the techniques described in

Wurzner, et al., *Complement Inflamm* 8:328–340, 1991. Preferably, the anti-C5 antibodies are used to treat GN in an amount effective to reduce the C5 convertase activity available in the patient's blood by at least about 50%.

In a particularly preferred embodiment of the invention, the anti-C5 antibodies are not immunoreactive against epitopes on the beta chain, but rather are immunoreactive against epitopes within the alpha chain of purified human complement component C5. In this embodiment the antibodies are also capable of blocking the conversion of C5 into C5a and C5b by C5 convertase. In an especially preferred example of this embodiment they can provide this blockade at substantially the same concentrations needed to block hemolytic activity.

Within the alpha chain, the most preferred antibodies bind to an amino-terminal region, however, they do not bind to free C5a. Particularly preferred targets for these antibodies within the alpha chain include the 5G46k fragment, the 5G27k fragment, the 5G325aa peptide, the 5G200aa peptide, or the KSSKC epitope. The scope of the invention also includes the 5G46k fragment, the 5G27k fragment, the 5G325aa peptide, the 5G200aa peptide, or the KSSKC epitope (SEQ ID NO:1) that are useful as immunogens and screening ligands for producing the antibodies of the invention.

Hybridomas producing monoclonal antibodies reactive with complement component C5 can be obtained according to the teachings of Sims, et al., U.S. Pat. No. 5,135,916. As discussed therein, antibodies are prepared using purified components of the complement membrane attack complex as immunogens. In accordance with the present invention, complement component C5 or C5b is preferably used as the immunogen. In accordance with a particularly preferred aspect of the present invention, the immunogen is the alpha chain of C5. Within the alpha chain, the most preferred immunogens include the 5G46k fragment, the 5G27k fragment, the 5G325aa peptide, or the 5G200aa peptide. A less preferred immunogen is the KSSKC epitope.

In accordance with the invention, the antibodies of the invention all share certain required functional properties. These are the ability to substantially inhibit complement hemolytic activity and to substantially inhibit the conversion of C5 to produce C5a. Preferably, but not requisitely, they provide these functions when used at a molar ratio of antibody to antigen (C5) of 3:1 or less.

A particularly preferred antibody of the invention is the 5G1.1 antibody (5G1.1, produced by the 5G1.1 hybridoma, ATCC designation HB-11625). Other particularly preferred antibodies of the present invention share the required functional properties discussed in the preceding paragraph and have any of the following characteristics:

(1) they compete with 5G1.1 for binding to portions of C5—the C5 alpha chain, the 5G46k fragment, the 5G27k fragment, the 5G325aa peptide (SEQ ID NO:1), the 5G200aa" peptide, or the KSSKC peptide—that are specifically immunoreactive with 5G1.1; and

(2) they specifically bind to the C5 alpha chain, the 5G46k fragment, the 5G27k fragment, the 5G325aa peptide, the 5G200aa" peptide, and/or the KSSKC peptide (SEQ ID NO:1). Such specific binding, and competition for binding can be determined by various methods well known in the art, including the plasmon surface resonance method (Johne et al., J. Immunol. Meth. 1993, 160:191–198).

(3) they block the binding of C5 to either C3 or C4 (which are components of C5 convertase).

Also in accordance with the invention, the antibodies preferably should prevent the cleavage of C5 to form C5a and C5b, thus preventing the generation of the anaphylatoxic activity associated with C5a and preventing the assembly of the membrane attack complex associated with C5b. In a particularly preferred embodiment, these anti-C5 antibodies will not impair the opsonization function associated with the activation of complement component C3 by a C3 convertase. Plasma C3 convertase activity can be measured by assaying plasma for the presence of C3a as described below under the heading "Histology." Preferably, the anti-C5 antibody produces essentially no reduction in plasma C3a levels.

General methods for the immunization of animals (in this case with C5 or C5b or another preferred immunogen), isolation of polyclonal antibodies or antibody producing cells, fusion of such cells with immortal cells (e.g., myeloma cells) to generate Hybridomas secreting monoclonal antibodies, screening of hybridoma supernatants for reactivity of secreted monoclonal antibodies with a desired antigen (in this case C5 or C5b or another preferred immunogen), the preparation of quantities of such antibodies in hybridoma supernatants or ascites fluids, and for the purification and storage of such monoclonal antibodies, can be found in numerous publications. These include: Coligan, et al., eds. *Current Protocols In Immunology*, John Wiley & Sons, New York, 1992; Harlow and Lane, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, 1988; Liddell and Cryer, *A Practical Guide To Monoclonal Antibodies*, John Wiley & Sons, Chichester, West Sussex, England, 1991; Montz, et al., *Cellular Immunol.* 127:337–351, 1990; Wurzner, et al., *Complement Inflamm.* 8:328–340, 1991; and Mollnes, et al., *Scand. J. Immunol.* 28:307–312, 1988.

As used herein, the term "antibodies" refers to immunoglobulins produced in vivo, as well as those produced in vitro by a hybridoma, and antigen binding fragments (e.g., Fab' preparations) of such immunoglobulins, as well as to recombinantly expressed antigen binding proteins, including immunoglobulins, chimeric immunoglobulins, "humanized" immunoglobulins, antigen binding fragments of such immunoglobulins, single chain antibodies, and other recombinant proteins containing antigen binding domains derived from immunoglobulins. As used herein, "antibodies" also refers to antigen binding synthetic peptides comprising sequences derived from the sequences of immunoglobulin antigen binding domains. As used herein, the term "recombinant mAbs" refers to recombinantly expressed antigen binding proteins. As used herein, the term "antibody-antigen binding site" refers to an antigen binding site of an antibody comprising at least one CDR sequence.

Antibodies whose amino acid sequences are full length immunoglobulin sequences that have not been truncated (e.g., to produce an scFv or an Fab) or mutated (e.g., spliced to form a chimeric antibody or humanized) are referred to herein as "native" antibodies. Publications describing methods for the preparation of such antibodies, in addition to those listed immediately above, include: Reichmann, et al., *Nature,* 332:323–327, 1988; Winter and Milstein, *Nature,* 349:293–299, 1991; Clackson, et al., *Nature,* 352:624–628, 1991; Morrison, *Annu Rev Immunol,* 10:239–265, 1992; Haber, *Immunol Rev,* 130:189–212, 1992; and Rodrigues, et al., *J Immunol,* 151:6954–6961, 1993.

While treatment of GN in accordance with the process of the present invention may be carried out using polyclonal or monoclonal antibodies, monospecific antibodies are preferred. As used herein "monospecific antibodies" refer to

antibodies that bind to a specific region of a particular antigen. All monoclonal antibodies are monospecific, but polyclonal antibodies are typically not monospecific.

As is known in the art, however, monospecific polyclonal antibodies may be prepared by various methods. For example, a peptide (e.g., an oligopeptide—as used hereinafter and in the claims, a polymer of 5 to 200 amino acids) may be used as an immunogen. Another procedure allowing the preparation of monospecific polyclonal antibodies is the use of antigen affinity purification techniques to isolate a monospecific antibody population from a polyclonal antibody mixture. In accordance with the present invention, peptides are preferred as immunogens for the production and as affinity ligands for the purification of monospecific polyclonal anti-KSSKC antibodies.

The native (i.e., non-engineered) monoclonal antibodies of the invention are preferably prepared by conventional means, with the 5G46k fragment, the 5G27k fragment, the 5G200aa peptide, the 5G325aa peptide, and/or the KSSKC peptide (SEQ ID NO:1) (e.g., immobilized on a polypropylene membrane as described below in Example 13) being used as screening ligand(s). This involves testing hybridoma supernatants for binding to each screening ligand.

In one preferred embodiment, the native mAbs of the invention are prepared using the alpha chain of human C5, or fragments thereof, as immunogen. Preferred fragments of the alpha chain of human C5 for this purpose include the 5G46k fragment, the 5G27k fragment, and/or the 5G200aa fragment. Although less preferred, the KSSKC peptide (SEQ ID NO:1) may also be used as an immunogen.

Another (albeit less preferred) immunogen and screening ligand for the preparation of antibodies within the scope of the novel antibodies of the present invention is the "cleavage site peptide," i.e., the peptide spanning amino acids 725 through 754 of SEQ ID NO:2 (the C5a cleavage site), as discussed below in Example 13.

In another preferred embodiment of the invention, the native mAbs of the invention are prepared in transgenic mice expressing human immunoglobulins (see, for example, Green et al., Nature Genet. 1994, 7:13–21). In this case, the same preferred immunogens and screening ligands are used as described for the preparation of other native mAbs.

In another preferred embodiment of the invention, the recombinant mAbs of the invention are prepared by screening phage display libraries expressing recombinant mab-encoding polynucleotides (preferably encoding human recombinant mabs). See, for example, Ames et al., 1994, supra; Smith and Scott, Meth. Enzymol. 1993, 217:228; Kay et al., Gene, 1993, 128:59–65. This screening is carried out with the screening ligands described above for the preparation of native mAbs. The recombinant mAbs of the invention are prepared by subcloning the recombinant mAb-encoding polynucleotides into a suitable expression vector, expressing them in a suitable host (as described below), and isolating the recombinant mAbs.

The present invention provides recombinant expression vectors which include the synthetic, genomic, or cDNA-derived nucleic acid fragments of the invention, i.e. polynucleotides encoding the mabs of the invention. The nucleotide sequence coding for any of the mAbs of the invention can be inserted into an appropriate expression vector, i.e., a vector that contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and translational signals can also be supplied by the native or source gene and/or its flanking regions.

A variety of host vector systems may be utilized to express the recombinant expression vectors of the invention. These include, but are not limited to, mammalian cell systems infected with recombinant virus (e.g., vaccinia virus, adenovirus, retroviruses, etc.); mammalian cell systems transfected with recombinant plasmids; insect cell systems infected with recombinant virus (e.g., baculovirus); microorganisms such as yeast containing yeast expression vectors, or bacteria transformed with recombinant bacteriophage DNA, recombinant plasmid DNA, or cosmid DNA (see, for example, Goeddel, 1990).

Useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well-known cloning vector pBR322 (American Type Culture Collection-"ATCC"-, 10801 University Boulevard, Manassas, Va. 20110-2209, United States of America; ATCC Accession No. 37017). These pBR322 "backbone sections," or functionally equivalent sequences, are combined with an appropriate promoter and the structural gene to be expressed. Promoters commonly used in recombinant microbial expression vectors include, but are not limited to, the lactose promoter system (Chang, et al., Nature 275:615), the tryptophan (trp) promoter (Goeddel, et al., 1980, Gene Expression Technology, Volume 185. Academic Press, Inc., San Diego, Calif.) and the tac promoter, or a fusion between the tac and trp promoters referred to as the trc promoter (Maniatis, 1982, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). Particularly preferred promoters include the T7 promoter, which is used in conjunction with host cell expression of a T7 RNA polymerase (see Studier et al. 1990, Meth. Enzymol. 185:60–89), and the trc promoter, which is found in several commercially available vectors, as described below.

Preferred bacterial expression vectors include, but are not limited to, the pET vectors (see Studier et al. 1990, supra) and the Trc vectors. Many of the pET vectors are commercially available from Stratagene Cloning Systems (La Jolla, Calif.). A particularly preferred vector is the pET Trc SO5/NI vector described below (SEQ ID NO:18). A Trc vector, pTrc 99A, is available from Pharmacia. Other Trc vectors include the pSE vectors (Invitrogen, San Diego, Calif.).

Preferred bacteria for expression of recombinant mAbs include *Bacillus subtilis* and, most preferably, *Escherichia coli*. A particularly preferred strain of *E. coli* is strain W3110 (ATCC designation 27325). Under certain unusual conditions it may be necessary to use standard bacterial genetics methods to prepare derivative strains of W3110, for example, when a contaminating bacteriophage ("phage") is present in the laboratory where the bacterial manipulations are being carried out. Generally, and particularly for large scale preparation of the recombinant anti-KSSKC mAbs of the invention, it is preferred to use unmodified W3110, or another fully characterized strain.

In cases where phage contamination is a problem and disinfection is not practicable or desirable, it is preferred to identify the phage contaminant and to then use a fully characterized bacterial strain having a known mutation rendering the bacterium resistant to the phage. Preferably the mutation is a null mutant for the receptor for the phage. In some instances, however, the generation use of a relatively uncharacterized phage-resistant derivative strain may be acceptable, particularly in small scale experimental work. When such derivative strains are desired, they may be prepared using the methods described below in Example 11.

For most purposes the use of unmodified W3110 or another fully characterized bacterial strain is generally preferred. This is particularly true for the preparation of phar-

maceutical agents comprising the recombinant anti-KSSKC mAbs of the invention. This is because of the problems, well known in the art, of using bacterial strains containing uncharacterized or partially characterized mutations for the production of ingredients of pharmaceutical agents.

The recombinant mAbs of the invention may also be expressed in fungal hosts, preferably yeast of the Saccharomyces genus such as *S. cerevisiae*. Fungi of other genera such as Aspergillus, Pichia or Kluyveromyces may also be employed. Fungal vectors will generally contain an origin of replication from the 2 μm yeast plasmid or another autonomously replicating sequence (ARS), a promoter, DNA encoding a mAb of the invention, sequences directing polyadenylation and transcription termination, and a selectable marker gene. Preferably, fungal vectors will include an origin of replication and selectable markers permitting transformation of both *E. coli* and fungi.

Suitable promoter systems in fungi include the promoters for metallothionein, 3-phosphoglycerate kinase, or other glycolytic enzymes such as enolase, hexokinase, pyruvate kinase, glucokinase, the glucose-repressible alcohol dehydrogenase promoter (ADH2), the constitutive promoter from the alcohol dehydrogenase gene, ADH1, and others. See, for example, Schena, et al. 1991 Meth. Enzymol. 194:389–398. Secretion signals, such as those directing the secretion of yeast alpha-factor or yeast invertase, can be incorporated into the fungal vector to promote secretion of a soluble recombinant mAb into the fungal growth medium. See Moir, et al., 1991, Meth. Enzymol. 194:491–507.

Preferred fungal expression vectors can be assembled using DNA sequences from pBR322 for selection and replication in bacteria, and fungal DNA sequences, including the ADH1 promoter and the alcohol dehydrogenase ADH1 termination sequence, as found in vector pAAH5 (Ammerer, 1983, Meth. Enzymol. 101:192). The ADH1 promoter is effective in yeast in that ADH1 mRNA is estimated to be 1–2% of total poly(A) RNA.

Various mammalian or insect cell culture systems can be employed to express recombinant mAbs. Suitable baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow, et al., 1988. Examples of suitable mammalian host cell lines include the COS cell of monkey kidney origin, mouse L cells, murine C127 mammary epithelial cells, mouse Balb/3T3 cells, Chinese hamster ovary cells (CHO), human 293 EBNA and HeLa cells, myeloma, and baby hamster kidney (BHK) cells, with myeloma cells, CHO cells, and human 293 EBNA cells being particularly preferred.

Mammalian expression vectors may comprise nontranscribed elements such as origin of replication, a suitable promoter and enhancer linked to the recombinant mAb gene to be expressed, and other 5' or 3' flanking sequences such as ribosome binding sites, a polyadenylation sequence, splice donor and acceptor sites, and transcriptional termination sequences.

The transcriptional and translational control sequences in mammalian expression vector systems to be used in transforming vertebrate cells may be provided by viral sources. For example, commonly used promoters and enhancers are derived from Polyoma virus, Adenovirus, Simian Virus 40 (SV40), and human cytomegalovirus, including the cytomegalovirus immediate-early gene 1 promoter and enhancer (CMV).

Particularly preferred eukaryotic vectors for the expression of recombinant anti-KSSKC mAbs are pAPEX-1 (SEQ ID NO:3 and, more preferably, pAPEX-3p, SEQ ID NO:4. The vector pAPEX-1 is a derivative of the vector pcDNAI/Amp (Invitrogen) which was modified to increase protein expression levels. First, the 3'-untranslated SV40 small-t antigen intron was removed by deletion of a 601 base pair XbaI/HpaI fragment since this intron is susceptible to aberrant splicing into upstream coding regions (Evans and Scarpulla, 1989 Gene 84:135; Huang and Gorman, 1990, Molec. Cell Biol. 10:1805). Second, a chimeric adenovirus-immunoglobulin hybrid intron was introduced into the 5'-untranslated region by replacing a 484 base pair NdeI-NotI fragment with a corresponding 845 base pair NdeI-NotI fragment from the vector pRc/CMV7SB (Sato et al., 1994, J. Biol. Chem. 269:17267). Finally, to increase plasmid DNA yields from *E. coli*, the resulting CMV promoter expression cassette was shuttled into the vector pGEM-4Z (Promega Corp. Madison, Wis.).

The vector pAPEX-3 is a derivative of the vector pDR2 (Clontech Laboratories, Inc. Palo Alto, Calif.) in which the EBNA gene was first removed by deletion of a 2.4 kb ClaI/AccI fragment. The RSV promoter was then replaced with the CMV promoter and the adenovirus/immunoglobulin chimeric intron by exchanging a 450 bp MluI/BamHI fragment from pDR2 with a 1.0 kb MluI/BamHI fragment from the vector pAPEX-1. For construction of pAPEX-3P, a 1.7 kb BstBI/SwaI fragment containing the HSV tk promoter and hygromycin phosphotransferase (hyg) gene was removed from pAPEX-3 and replaced with a 1.1 kb SnaBI/NheI fragment containing the SV40 early promoter and puromycin acetyltransferase (pac) gene (Morgenstern and Land, 1990, Nucleic Acids Res. 18:3587–3596) plus a 137 bp XbaI/ClaI fragment containing an SV40 polyadenylation signal from the vector pAPEX-1.

A particularly preferred host cell for the expression of recombinant mAb-encoding inserts in the pAPEX vectors is the human 293 EBNA cell line (Invitrogen, San Diego, Calif.).

Another preferred eukaryotic vector for the expression of recombinant mAbs is pcDNAI/Amp (Invitrogen Corporation, San Diego, Calif.). The pcDNAI/Amp expression vector contains the human cytomegalovirus immediate-early gene I promoter and enhancer elements, the Simian Virus 40 (SV40) consensus intron donor and acceptor splice sequences, and the SV40 consensus polyadenylation signal. This vector also contains an SV40 origin of replication that allows for episomal amplification in cells (e.g., COS cells, MOP8 cells, etc.) transformed with SV40 large T antigen, and an ampicillin resistance gene for propagation and selection in bacterial hosts.

Purified recombinant mAbs are prepared by culturing suitable host/vector systems to express the recombinant mAb translation products of the nucleic acid molecules of the present invention, which are then purified from the culture media or cell extracts of the host system, e.g., the bacteria, insect cells, fungal, or mammalian cells. Fermentation of fungi or mammalian cells that express recombinant mAb proteins containing a histidine tag sequence (a sequence comprising a stretch of at least 5 histidine residues) as a secreted product greatly simplifies purification. Such a histidine tag sequence enables binding under specific conditions to metals such as nickel, and thereby to nickel (or other metal) columns for purification. Recombinant mAbs may also be purified by protein G affinity chromatography (Proudfoot et al., 1992, Protein Express. Purif. 3:368).

Additional preferred embodiments are numbered and set forth below as "favored embodiments."

FAVORED EMBODIMENTS

1. A method for the treatment of glomerulonephritis in a patient in need of such treatment comprising introducing an

antibody that binds to complement component C5 into the patient's bloodstream in an amount effective to substantially reduce the cell-lysing ability of complement present in the patient's blood.

2. The method of favored embodiment 1 wherein the antibody reduces the conversion of complement component C5 into complement components C5a and C5b.

3. The method of favored embodiment 1 wherein the antibody binds to C5b.

4. The method of favored embodiment 1 wherein the antibody does not substantially inhibit formation of complement component C3b.

5. The method of favored embodiment 1 wherein the antibody is introduced into the patient's bloodstream in a dose that is not greater than 0.1 grams per kilogram.

6. An article of manufacture comprising packaging material and a pharmaceutical agent contained within said packaging material, wherein:

(a) said pharmaceutical agent comprises an antibody to complement component C5, said antibody being effective in substantially reducing the cell-lysing ability of complement present in the patient's blood; and

(b) said packaging material comprises a label which indicates that said pharmaceutical agent is for use in the treatment of kidney disease.

7. The article of manufacture of favored embodiment 6 wherein the label indicates that said pharmaceutical agent is for use in the treatment of nephritis.

8. The article of manufacture of favored embodiment 7 wherein the label indicates that said pharmaceutical agent is for use in the treatment of glomerulonephritis.

9. The article of manufacture of favored embodiment 6 wherein the pharmaceutical agent is to be used at a dosage level not greater than 0.1 grams per kilogram.

10. An antibody comprising at least one antibody-antigen binding site, said antibody exhibiting specific binding to human complement component C5, said specific binding being targeted to the alpha chain of human complement component C5, wherein the antibody inhibits complement activation in a human body fluid and does not specifically bind to the human complement activation product free C5a.

11. The antibody of favored embodiment 10 wherein the inhibition of complement activation in the human body fluid is measurable as a substantial increment of blockade of C5a generation and a substantial increment of blockade of complement hemolytic activity in the body fluid, said increment of blockade of C5a generation being substantially equal to said increment of blockade of complement hemolytic activity.

12. The antibody of favored embodiment 10 wherein, upon binding to human C5, the antibody substantially inhibits the ability of C5 to bind to human complement component C3.

13. The antibody of favored embodiment 10 wherein, upon binding to human C5, the antibody substantially inhibits the ability of C5 to bind to human complement component C4.

14. The antibody of favored embodiment 10 wherein the antibody binds specifically with a 5G46k fragment.

15. The antibody of favored embodiment 10 wherein the antibody binds specifically to a 5G27k fragment.

16. The antibody of favored embodiment 10 wherein the antibody binds specifically to a 5G325aa peptide.

17. The antibody of favored embodiment 10 wherein the antibody binds specifically to a 5G200aa peptide.

18. The antibody of favored embodiment 10 wherein the antibody binds specifically to a KSSKC peptide.

19. The antibody of favored embodiment 10 wherein the inhibition of complement activation in the human body fluid is measurable as a substantially complete blockade of C5a generation in the body fluid and a substantially complete blockade of complement hemolytic activity in the body fluid when the antibody is added to the body fluid at a concentration yielding a ratio equal to or less than 10 moles of antibody-antigen binding sites of the antibody to 1 mole of human C5 in the body fluid.

20. The antibody of favored embodiment 19 wherein the concentration yields a ratio equal to or less than 3 moles of antibody-antigen binding sites of the antibody to 1 mole of human C5 in the body fluid.

21. Hybridoma 5G1.1 having ATCC designation HB-11625.

22. An antibody produced by the hybridoma of favored embodiment 21.

23. An antibody that can compete with the antibody of favored embodiment 22 for binding to the alpha chain of human C5.

24. A nucleic acid molecule comprising a nucleotide sequence encoding an scFv polypeptide comprising an amino acid sequence corresponding to amino acid 1 through amino acid 248 of SEQ ID NO:7.

25. A nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising a variable light chain region amino acid sequence corresponding to amino acid 3 through amino acid 110 of SEQ ID NO:9.

26. A nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:10.

27. An isolated protein comprising:

(a) a first polypeptide region comprising a variable light chain region amino acid sequence corresponding to amino acid 3 through amino acid 110 of SEQ ID NO:9.; and

(b) a second polypeptide region comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:10.

28. An isolated polypeptide comprising an amino acid sequence encoded by the nucleic acid molecule of favored embodiment 24, favored embodiment 25, or favored embodiment 26, wherein the polypeptide is an antibody.

29. A nucleic acid vector comprising a first nucleic acid molecule covalently and operatively linked to a second nucleic acid molecule so that a host containing the vector expresses the polypeptide coded for by the first nucleic acid molecule, wherein the first nucleic acid molecule is the nucleic acid molecule of favored embodiment 24, favored embodiment 25, or favored embodiment 26.

30. A recombinant host cell containing the nucleic acid vector of favored embodiment 29.

31. A method for producing an isolated C5 antibody polypeptide comprising growing the recombinant host cell of favored embodiment 30 such that the polypeptide encoded by the first nucleic acid molecule of the vector is expressed by the host cell, and isolating the expressed polypeptide, wherein the expressed polypeptide is an anti-C5 antibody.

32. The isolated anti-C5 antibody of favored embodiment 31.

33. A nucleic acid molecule comprising a nucleotide sequence encoding an scFv comprising an amino acid sequence corresponding to amino acid 1 through amino acid 248 of SEQ ID NO:8.

34. A nucleic acid molecule comprising a nucleotide sequence encoding an scFv comprising an amino acid sequence corresponding to amino acid 1 through amino acid 248 of SEQ ID NO:17.

35. A nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising a variable light chain region amino acid sequence corresponding to amino acid 1 through amino acid 108 of SEQ ID NO:15.

36. A nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising a variable light chain region amino acid sequence corresponding to amino acid 3 through amino acid 110 of SEQ ID NO:14.

37. A nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:16.

38. A nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:12.

39. A nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:11.

40. An isolated protein comprising:

(a) a first polypeptide region comprising a variable light chain region amino acid sequence corresponding to amino acid 1 through amino acid 108 of SEQ ID NO:15; and

(b) a second polypeptide region comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:16.

41. An isolated protein comprising:

(a) a first polypeptide region comprising a variable light chain region amino acid sequence corresponding to amino acid 1 through amino acid 1 through amino acid 108 of SEQ ID NO:15; and

(b) a second polypeptide region comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:12.

42. An isolated protein comprising:

(a) a first polypeptide region comprising a variable light chain region amino acid sequence corresponding to amino acid 1 through amino acid 108 of SEQ ID NO:15; and

(b) a second polypeptide region comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:11.

43. An isolated protein comprising:

(a) a first polypeptide region comprising a variable light chain region amino acid sequence corresponding to amino acid 3 through amino acid 110 of SEQ ID NO:14; and

(b) a second polypeptide region comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:16.

44. An isolated protein comprising:

(a) a first polypeptide region comprising a variable light chain region amino acid sequence corresponding to amino acid 3 through amino acid 110 of SEQ ID NO:14; and

(b) a second polypeptide region comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:12.

45. An isolated protein comprising:

(a) a first polypeptide region comprising a variable light chain region amino acid sequence corresponding to amino acid 3 through amino acid 110 of SEQ ID NO:14; and

(b) a second polypeptide region comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:11.

46. An isolated protein comprising the amino acid sequence encoded by the nucleic acid molecule of favored embodiment 33, favored embodiment 34, favored embodiment 35, favored embodiment 36, favored embodiment 37, favored embodiment 38, or favored embodiment 39, wherein the isolated protein is an anti-C5 antibody.

47. A nucleic acid vector comprising a first nucleic acid molecule covalently and operatively linked to a second nucleic acid molecule so that a host containing the vector expresses the polypeptide coded for by the first nucleic acid molecule, wherein the first nucleic acid molecule is the nucleic acid molecule of favored embodiment 33, favored embodiment 34, favored embodiment 35, favored embodiment 36, favored embodiment 37, favored embodiment 38, or favored embodiment 39.

48. A recombinant host cell containing the nucleic acid vector of favored embodiment 47.

49. A method for producing an isolated anti-C5 antibody protein comprising growing the recombinant host cell of favored embodiment 48 such that a protein encoded by the nucleic acid molecule is expressed by the host cell, and isolating the expressed protein, wherein the expressed protein is an anti-C5 antibody.

50. The isolated anti-C5 antibody of favored embodiment 47.

51. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable light region CDR3 comprising an amino acid sequence corresponding to amino acid 93 through amino acid 98 of SEQ ID NO:7;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

52. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable light region CDR3 comprising an amino acid sequence corresponding to amino acid 91 through amino acid 99 of SEQ ID NO:8;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

53. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable heavy region CDR1 comprising an amino acid sequence corresponding to amino acid 156 through amino acid 159 of SEQ ID NO:7;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

54. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable heavy region CDR1 comprising an amino acid sequence corresponding to amino acid 152 through amino acid 161 of SEQ ID NO:8;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

55. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable heavy region CDR2 comprising an amino acid sequence cor-

responding to amino acid 179 through amino acid 182 of SEQ ID NO:7;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

56. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable heavy region CDR2 comprising an amino acid sequence corresponding to amino acid 176 through amino acid 186 of SEQ ID NO:8;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

57. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable heavy region CDR3 comprising an amino acid sequence corresponding to amino acid 226 through amino acid 236 of SEQ ID NO:7;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

58. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable heavy region CDR3 comprising an amino acid sequence corresponding to amino acid 225 through amino acid 237 of SEQ ID NO:8;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

59. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable light region CDR3 comprising an amino acid sequence corresponding to amino acid 91 through amino acid 99 of SEQ ID NO:8;

(b) a nucleotide sequence encoding a variable heavy region CDR1 comprising an amino acid sequence corresponding to amino acid 152 through amino acid 161 of SEQ ID NO:8;

(c) a nucleotide sequence encoding a variable heavy region CDR2 comprising an amino acid sequence corresponding to amino acid 176 through amino acid 186 of SEQ ID NO:8; and

(d) a nucleotide sequence encoding a variable heavy region CDR3 comprising an amino acid sequence corresponding to amino acid 225 through amino acid 237 of SEQ ID NO:8.

60. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable light region CDR3 comprising an amino acid sequence corresponding to amino acid 91 through amino acid 99 of SEQ ID NO:8;

(b) a nucleotide sequence encoding a variable heavy region CDR1 comprising an amino acid sequence corresponding to amino acid 152 through amino acid 161 of SEQ ID NO:8;

(c) a nucleotide sequence encoding a variable heavy region CDR2 comprising an amino acid sequence corresponding to amino acid 176 through amino acid 192 of SEQ ID NO:8; and

(d) a nucleotide sequence encoding a variable heavy region CDR3 comprising an amino acid sequence corresponding to amino acid 225 through amino acid 237 of SEQ ID NO:8.

61. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable light region CDR3 comprising an amino acid sequence corresponding to amino acid 91 through amino acid 99 of SEQ ID NO:8;

(b) a nucleotide sequence encoding a variable heavy region CDR1 comprising an amino acid sequence corresponding to amino acid 152 through amino acid 161 of SEQ ID NO:8;

(c) a nucleotide sequence encoding a variable heavy region CDR2 comprising an amino acid sequence corresponding to amino acid 179 through amino acid 182 of SEQ ID NO:7; and

(d) a nucleotide sequence encoding a variable heavy region CDR3 comprising an amino acid sequence corresponding to amino acid 225 through amino acid 237 of SEQ ID NO:8.

62. An isolated protein comprising the amino acid sequence encoded by the nucleic acid molecule of favored embodiment 51, favored embodiment 52, favored embodiment 53, favored embodiment 54, favored embodiment 55, favored embodiment 56, favored embodiment 57, favored embodiment 58, favored embodiment 59, favored embodiment 60 or favored embodiment 87.

63. The isolated protein of favored embodiment 62 wherein the protein is an anti-C5 antibody.

64. A nucleic acid vector comprising a first nucleic acid molecule, said first nucleic acid molecule corresponding to the nucleic acid molecule of favored embodiment 51, favored embodiment 52, favored embodiment 53, favored embodiment 54, favored embodiment 55, favored embodiment 56, favored embodiment 57, favored embodiment 58, or favored embodiment 87 covalently and operatively linked to a second nucleic acid molecule so that a host containing the vector expresses the protein encoded by the first nucleic acid molecule.

65. A recombinant host cell containing the nucleic acid vector of favored embodiment 64.

66. A method for producing an anti-C5 antibody comprising growing the recombinant host cell of favored embodiment 65 so that the protein encoded by the nucleic acid molecule is expressed by the host cell, and isolating the expressed protein, wherein the expressed protein is an anti-C5 antibody.

67. The anti-C5 antibody of favored embodiment 66.

68. An isolated 5G46k fragment of human complement component C5.

69. An isolated 5G27k fragment of human complement component C5.

70. An isolated 5G325aa peptide.

71. An isolated 5G200aa peptide.

72. An isolated oligopeptide comprising an amino acid sequence corresponding to amino acid 8 through amino acid 12 of SEQ ID NO:1, i.e., Lys Ser Ser Lys Cys, or in single letter notation, KSSKC.

73. A method of inducing an animal to produce an anti-C5 antibody comprising repeatedly immunizing an animal with the isolated alpha chain of human C5.

74. A method of inducing an animal to produce an anti-C5 antibody comprising immunizing an animal with the isolated 5G46k fragment of favored embodiment 68.

75. A method of inducing an animal to produce an anti-C5 antibody comprising immunizing an animal with the isolated 5G27k fragment of favored embodiment 69.

76. A method of inducing an animal to produce an anti-C5 antibody comprising immunizing an animal with the isolated 5G325aa peptide of favored embodiment 70.

77. A method of inducing an animal to produce an anti-C5 antibody comprising immunizing an animal with the isolated 5G200aa peptide of favored embodiment 71.

78. A method of inducing an animal to produce an anti-C5 antibody comprising immunizing an animal with the isolated oligopeptide of favored embodiment 72.

79. A method of identifying an anti-C5 antibody comprising screening candidate antibodies with the isolated alpha chain of human C5.

80. A method of identifying an anti-C5 antibody comprising screening candidate antibodies with the isolated 5G46k fragment of favored embodiment 68.

81. A method of identifying an anti-C5 antibody comprising screening candidate antibodies with the isolated 5G27k fragment of favored embodiment 69.

82. A method of identifying an anti-C5 antibody comprising screening candidate antibodies with the isolated 5G325aa peptide of favored embodiment 70.

83. A method of identifying an anti-C5 antibody comprising screening candidate antibodies with the isolated 5G200aa peptide of favored embodiment 71.

84. A method of identifying an anti-C5 antibody comprising screening candidate antibodies with the isolated oligopeptide of favored embodiment 72.

85. A method of treating a patient in need of complement inhibition comprising administering the antibody of favored embodiment 10, favored embodiment 22, favored embodiment 23, favored embodiment 28, favored embodiment 32, favored embodiment 46, favored embodiment 50, favored embodiment 63, or favored embodiment 67 to the patient in an amount effective to substantially reduce hemolytic activity in a body fluid of the patient.

86. The antibody of favored embodiment 10 wherein the antibody is a recombinant antibody that comprises a human constant domain.

87. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable heavy region CDR2 comprising an amino acid sequence corresponding to amino acid 176 through amino acid 192 of SEQ ID NO:8;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

88. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable heavy region CDR2 comprising an amino acid sequence corresponding to amino acid 176 through amino acid 192 of SEQ ID NO:8;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

89. An isolated antibody comprising any one of the CDR regions of CO12, CO13, CO14, Co15, DO12b, DO12C, DO12D.

90. An isolated nucleic acid molecule encoding the antibody of favored embodiment 89.

EXAMPLES

Without intending to limit it in any manner, the present invention will be more fully described by the following examples. The methods and materials which are common to various of the examples are as follows.

Induction of GN in Mice

Four month old female B10.D2/nSnJ mice averaging approximately 25 gms each were obtained from the Jackson Laboratory, Bar Harbor, Me. Mice were injected with 0.1 mL daily (six days per week) of a 40 mg/mL solution of horse apoferritin (HAF), which was prepared by dilution of a saline solution of HAF (Sigma Chemical Company Catalog No. A-3641) with PBS.

Anti-C5 Monoclonal Antibodies

Monoclonal antibodies that bind to complement component C5 of the mouse were prepared by standard methods as an IgG fraction from supernatants of cultures of hybridoma BB5.1 (Frei, et al., 1987), which was obtained from Dr. Brigitta Stockinger of the National Institute for Medical Research, Mill Hill, London, England.

Histology

Kidneys were subjected to microscopic analysis using standard histochemical staining and immunofluorescence techniques. Periodic Acid Schiff (PAS) staining of 5 paraffin sections was by standard methods using a HARLECO PAS histochemical reaction set (EM Diagnostic Systems, Gibbstown, N.J., number 64945/93) according to the manufacturer's directions.

Immunofluorescence staining of 5μ cryostat sections was carried out by standard methods using FITC conjugated sheep anti-mouse C3 (Biodesign International, Kennebunk, Me, Catalog No. W90280F) to detect murine complement component C3, or FITC conjugated goat anti-mouse IgG, IgA, and IgM (Zymed Laboratories, South San Francisco, Calif., Catalog No. 65-6411) to detect immune complexes.

Urine Assays

Protein and glucose levels were determined by spotting urine samples on CHEMSTRIP 2GP dipsticks (Boehringer Mannheim Diagnostics, Indianapolis, Ind., Catalog No. 200743). The detection areas of these strips change color when exposed to urine containing protein or glucose; a lack of color change indicates no detectable protein or glucose is present. The level of analyte in the urine being tested is read out by matching changed colors with color charts supplied by the manufacturer. The urine protein chart shows colors corresponding to trace, 30, 100, and 500 mg/dL.

Cell Lysis Assays

The cell-lysing ability of complement in blood can be determined using hemolytic assays that are performed as follows: Chicken erythrocytes are washed well in GVBS (Rollins, et al., *J Immunol* 144:3478–3483, 1990, Sigma Chemical Co. St. Louis, Mo., catalog No. G-6514) and resuspended to 2×10^8/mL in GVBS. Anti-chicken erythrocyte antibody (IgG fraction of anti-chicken-RBC antiserum, Intercell Technologies, Hopewell, N.J.) is added to the cells at a final concentration of 25 μg/mL and the cells are incubated for 15 min. at 23° C. The cells are washed 2× with GVBS and 5×10^6 cells are resuspended to 30 μL in GVBS. A 100 μL volume of serum test solution is then added to yield a final reaction mixture volume of 130 μL. As used herein, reference to the serum percentage and/or serum input in these assays indicates the percent serum in the 100 μL volume of serum test solution.

For assays of mouse serum activity, the 100 μL volume of serum test solution contained 50 μL of diluted (in GVBS) mouse serum and 50 μL of human C5 deficient serum (Quidel Corporation, San Diego, Calif.). For assays of human serum activity, the serum test solution may contain up to 100% human plasma or serum, with hybridoma supernatants and/or GVBS being added to yield the 100 μL volume. For the assays used to screen hybridoma supernatants discussed below in Example 7, each 100 μL volume of serum test solution contained 50 μL of hybridoma supernatant and 50 μL of a 10% solution of human serum in GVBS, yielding a 5% human serum input.

After incubation for 30 min. at 37° C., percent hemolysis was calculated relative to a fully lysed control sample. Hemolysis was determined by spinning the cells down and measuring released hemoglobin in the supernatant as the optical density at 415 nm.

A 50% reduction in hemolysis after treatment with the anti-C5 antibodies used in the practice of the invention means that the percent hemolysis after treatment is one half of the percent hemolysis before treatment.

Example 1

Anti-C5 Antibodies Inhibit Glomerular Inflammation and Enlargement

This example illustrates that anti-C5 antibodies will inhibit glomerular inflammation and enlargement.

The protocol for these experiments was as follows. GN-induced mice were treated with anti-C5 antibodies or with PBS as a control after 2 weeks of GN induction. Each mouse received 750 μg of anti-C5 monoclonal antibodies in PBS (30 mg/kg in a 25 gm mouse) or an equal volume of PBS alone. The amount injected was from 0.25 to 0.365 mL (the concentration of antibodies in PBS varied), which was administered by intraperitoneal injection once a day, six days a week. After an additional 2 weeks of induction and treatment, the animals were sacrificed and kidneys were harvested and prepared for histological examination as described above. Kidneys were also obtained from age-matched uninduced and untreated control mice.

FIG. **1** shows sections of mouse kidneys with a single glomerulus located centrally amidst surrounding interstitium and cross sections of convoluted tubules in each section. As can be seen therein, the kidneys of the GN-induced, PBS-treated mice (FIG. **1**B) developed severe crescentic glomerular pathology, including inflammatory glomerular hypercellularity, apparent basement membrane thickening, and glomerular enlargement, while the glomeruli of the GN-induced, anti-C5-treated animals (FIG. **1**C) were essentially indistinguishable from the glomeruli of the normal healthy kidneys of the uninduced untreated mice (FIG. **1**A).

Note that in the glomeruli with severe crescentic pathology, the size of the glomerular capillary network (glomerular tuft) is not enlarged, but shows signs of compression by a crescentic-shaped proliferation of epithelial cells and PAS-positive material, and the Bowman's capsule is dramatically enlarged. Also note that in the section of diseased glomerulus shown in FIG. **1**B, the capillary network is split in half by a projection of the hypercellular crescentic mass.

The non-inflamed glomerulus of the uninduced untreated mouse shown in FIG. **1**A is approximately 100μ in diameter; the inflamed glomerulus of the GN-induced, PBS treated mouse shown in FIG. **1**B is approximately 175μ in diameter; the non-inflamed glomerulus of the GN-induced, anti-C5-treated mouse shown in FIG. **1**C is approximately 90μ in diameter.

Example 2

Anti-C5 Antibodies Prevent/Reduce Proteinuria Associated with GN

This example demonstrates that treatment with anti-C5 antibodies results in the prevention/reduction of kidney damage as evidenced by the lack of significant amounts of protein in the urine (i.e. the presence of less than 100 mg/dL of protein in the urine).

The protocol for the experiments of this example was the same as that used in the experiments of Example 1. Five PBS-treated, GN-induced mice, 6 anti-C5-treated, GN-induced mice, and 4 age-matched untreated uninduced mice were used in this study. A first set of urine samples was analyzed prior to treatment after the initial 2 week induction period. A second set of urine samples was analyzed after the 2 week treatment period. None of the untreated uninduced control animals had detectable protein in their urine at either of these timepoints.

The results obtained with the GN-induced mice are set forth in Table 1. As shown therein, at the end of the 2 week PBS treatment period, 4 out of the 5 PBS treated (control) animals developed significant proteinuria, i.e., at least 100 mg/dL of protein in the urine. The fifth animal (mouse D in Table 1) did not have detectable protein in the urine at either timepoint but, unlike the other mice in the study, was found to have very high levels of glucose in the urine after the 2 week PBS treatment period, suggesting that this animal was physiologically compromised.

In the anti-C5-treated, GN-induced group, the one mouse that developed significant proteinuria at the end of the initial 2 week induction period (mouse 6 in Table 1) improved by the end of the 2 week antibody treatment period. In addition, in contrast to the development of significant proteinuria in 4 out of 5 PBS-treated, GN-induced mice, none of the anti-C5-treated, GN-induced mice exhibited significant proteinuria at the end of the 2 week antibody treatment period.

Example 3

Anti-C5 Antibodies do not Inhibit Glomerular Immune Complex Deposition

This example demonstrates that anti-C5 antibodies used in the practice of the invention achieve their therapeutic effects even though immune complexes are deposited in the glomeruli of treated animals at equivalent levels to those seen in the glomeruli of PBS-treated animals. The example further illustrates that the mechanism of operation of the anti-C5 antibodies is not through the inhibition of immune complex deposition in the glomerulus.

The protocol used in the experiments of this example was the same as that used in the experiments of Example 1. Immunofluorescence staining as described above was performed on sections from the same kidneys harvested in Example 1.

The results are shown in FIG. **2**. As can be seen in this figure, equivalent amounts of immune complexes were deposited in the glomeruli of the kidneys of both the PBS-treated, GN-induced mice (FIG. **2**B) and the anti-C5-treated, GN-induced mice (FIG. **2**C), but not in the untreated uninduced controls (FIG. **2**A). Kidneys of GN-induced mice harvested after the 2 week induction period, but before treatment, showed immune complex deposits in the glomeruli, but at lower levels (as indicated by lower fluorescence intensity) than in the kidney sections shown in FIG. **2**B and FIG. **2**C.

Example 4

Anti-C5 Antibodies Inhibit C5b-9 Generation

This example demonstrates that the anti-C5 antibodies used in the practice of the invention inhibit C5b-9 generation. C5b-9 generation was assayed in 2 ways: (1) by testing the cell-lysing (hemolytic) ability of blood samples, and (2) by measuring levels of soluble C5b-9 in blood samples.

FIG. **3** shows the results of cell lysis assays performed as described above, with mouse serum added to the percentage indicated on the X axis ("serum input %"). In these assays, serum from GN-induced animals treated with either anti-C5 antibodies in PBS or PBS alone (see above) was assayed at the end of the two week treatment period. Serum from normal, uninduced, uninjected mice ("normal mouse serum") obtained from Sigma Chemical Company (St. Louis, Mo., Catalog No. S-3269) was also assayed as an additional control. These results indicate that the anti-C5

monoclonal antibody administered to mice at a dosage of 30 mg/Kg completely blocked the cell lysing ability of mouse blood at serum input levels 4-fold higher than the levels of normal serum that produce maximum hemolysis in the assay.

The effects of an anti-C5 monoclonal antibody raised to human C5 was evaluated in circulating human blood. Hybridoma N19/8 (Wurzner, et al., 1991) was obtained from Dr. Otto Götze, Department of Immunology, University of G öttingen, FRG. The C5 monoclonal antibody was prepared following immunization of mice with purified human C5 protein as described in Wurzner, et al., (1991). The hybridoma was propagated in mice, and the monoclonal antibody recovered and purified as an IgG fraction from mouse ascites fluid (*Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, 1988; *Current Protocols In Immunology*, John Wiley & Sons, New York, 1992).

To carry out these experiments, as well as others described below in Examples 5 and 6, 300 mL of whole human blood was drawn from a healthy human donor and additionally a 1 mL sample was removed as a control sample for later analysis. The blood was diluted to 600 mL by the addition of Ringer's lactate solution containing 10 U/mL heparin. The anti-C5 mAb (30 mg in sterile PBS) was added to the diluted blood to a final concentration of 50 μg/mL (results using test samples obtained in this way are labeled "+anti-C5 sample" in FIG. **4** and FIG. **6**). In a control experiment, an equal volume of sterile PBS was added to diluted blood (results using control samples obtained in this way are labeled "−anti-C5 sample" in FIG. **4** and FIG. **6**).

The blood was then used to prime the extracorporeal circuit of a COBE CML EXCEL membrane oxygenator cardiopulmonary bypass (CPB) machine (Cobe BCT, Inc., Lakewood, Colo.) and circulation through the circuit was started. The circuit was cooled to 28° C. and circulated for 60 minutes. The circuit was then warmed to 37° C. and circulated for an additional 30 minutes, after which time the experiment was terminated. Mechanical circulation of blood in this fashion activates the complement cascade. Samples were taken at several time points.

At each time point an aliquot of blood was taken, and subaliquots were centrifuged to remove all cells and the remaining plasma diluted 1:1 in QUIDEL sample preservation solution (Quidel Corporation, San Diego, Calif.) and stored at −80° C. for subsequent evaluation of soluble C5b-9 (sC5b-9) generation. Diluted subaliquots of plasma were also frozen for evaluation of C3a generation (see Example 5, below). Undiluted subaliquots of plasma were frozen at −80° C. for analysis in hemolytic assays to evaluate the pharmacokinetics of the effects of the anti-C5 antibodies on the cell lysing ability of complement present in the blood (see Example 6, below). These experiments are also discussed in copending U.S. patent application Ser. No. 08/217,391, filed Mar. 23, 1994, now U.S. Pat. No. 5,853,722.

sC5b-9 assays were performed before the addition of the antibody or the commencement of the CPB circuit (labeled "Pre Tx" in FIG. **4** and FIG. **6**) using undiluted blood (i.e. blood from the 1 mL sample taken before the blood was diluted with Ringer's lactate solution—labeled "undil" in FIG. **4** and FIG. **6**) and Ringer's lactate solution diluted blood (labeled "dil" in FIG. **4** and FIG. **6**). Samples of Ringer's lactate solution diluted blood to which the antibody had been added (labeled "Post Tx" in FIG. **4** and FIG. **6**) were assayed at the times indicated after starting the CPB circuit.

As can be seen in FIG. **4**, while sC5b-9 levels were more than 4-fold higher in untreated samples after 90 minutes of circulation than before circulation, the anti-C5 antibody completely inhibited C5b-9 generation throughout the 90 minute time course of circulation so that sC5b-9 levels during circulation were essentially equivalent to control, uncirculated samples, at all timepoints.

Example 5

Anti-C5 Antibodies do not Inhibit C3 Deposition or Activation

This example demonstrates that treatment with anti-C5 antibodies does not result in the inhibition of the activation of complement component C3 or in the deposition of C3 or its activated fragments in glomeruli.

The deposition of C3, or the fragments generated by its activation (e.g., C3a and C3b), in the glomeruli of GN-induced and GN-uninduced mice was visualized by immunofluorescence staining with a FITC-conjugated sheep anti-mouse C3 antibody preparation using standard methods, as described above. As can be seen in FIG. **5**, kidneys of the PBS-treated (FIG. **5**B) and the anti-C5 antibody-treated (FIG. **5**C) GN-induced mice had roughly equivalent levels of C3 immunoreactive material in the glomeruli, while the uninduced untreated control mice had only traces of C3 immunoreactive material in their kidneys (FIG. **5**A).

Note that the print shown in FIG. **5**A was overexposed compared to those of FIG. **5**B and FIG. **5**C to show the very slight levels of reactivity present in normal uninduced kidneys. Kidneys of GN-induced mice harvested after the 2 week induction period, but before treatment, showed C3 immunoreactive materials in the glomeruli, but at lower levels (as indicated by lower fluorescence intensity) than in the kidney sections shown in FIG. **5**B and FIG. **5**C.

Anti-human C5 antibodies were also tested for possible inhibition of C3 activation in human blood prepared and circulated as described above in Example 4. Activation of complement component C3 was indicated by the presence in the blood of the C3 activation product C3a. C3a assays were performed as follows.

The plasma samples that had previously been diluted in QUIDEL sample preservation solution and frozen (see Example 4) were assayed for the presence of C3a by using the QUIDEL C3A EIA kit (Quidel Corporation, San Diego, Calif.) according to the manufacturer's specifications. Concentrations of C3a in the samples is expressed as ng/well as determined by comparison to a standard curve generated from samples containing known amounts of human C3a.

As seen in FIG. **6**, the addition of the anti-C5 mAb had no inhibitory effect on the production of C3a during the circulation of human blood in this experiment.

Example 6

Pharmacokinetics of Anti-C5 Antibodies

The in vivo duration of action of mAb BB5.1, and a Fab' fragment of mAb BB5.1 (prepared by standard methods) was determined in normal female BALB/cByJ mice (averaging approximately 20 gms each) which were obtained from the Jackson Laboratory, Bar Harbor, Me. The mice were given a single intravenous injection (at 35 mg/kg body weight) of the mAB or the Fab' fragment of the mAb (or an equal volume of PBS as a control). Blood samples were collected from the retroorbital plexus at 1, 4, 24, 96, and 144 hours after administration of PBS; 4, 16, and 24 hours after administration of the Fab' fragment of mAb

BB5.1; and 4, 24, 48, 72, 96, and 144 hours after administration of intact mAb BB5.1.

FIG. 7A shows the time course of inhibition of the cell-lysing ability of complement in mouse blood (determined, by testing serum obtained from the blood and diluted to 2.5%, as described above) after the in vivo administration of the mAb, the Fab' fragment, or the PBS. As shown in the figure, the mAb almost completely inhibited the hemolytic activity of the blood throughout the 6 day test period. The Fab', however, had a half-life of approximately 24 hours.

In addition to the above experiments, at the end of the 6 day testing period all of the mice were sacrificed. Kidneys, lungs, and livers were harvested and examined by gross inspection, as well as by microscopic examination of stained sections. All of the organs of the anti-C5 antibody treated animals appeared the same as those taken from PBS control treated animals. The overall appearance of the test and control mice was also indistinguishable prior to necropsy.

Anti-human C5 antibodies were also tested for pharmacokinetic properties in circulating human blood as described above in Example 4. As described therein, the hemolysis inhibiting effects of an anti-human C5 monoclonal antibody were assayed over a 90 minute period of circulation. The results of these assays are charted in FIG. 7B, and show that the N19/8 anti-C5 mAb essentially completely inhibited the cell lysing ability of the human blood during the entire 90 minute period of circulation.

The results of these experiments demonstrate that the anti-C5 antibodies will survive in the bloodstream for a substantial period of time, thus making periodic administration practical.

Example 7

Preparation of Anti-C5 Monoclonal Antibodies

A monoclonal antibody suitable for use in the practice of the present invention was prepared in accordance with the teachings of Sims, et al., U.S. Pat. No. 5,135,916, as follows.

Balb/c mice were immunized three times by intraperitoneal injection with human C5 protein (Quidel Corporation, San Diego, Calif., Cat # A403). The first injection contained 100 μg of C5 protein in a complete Freund's adjuvant emulsion, the second immunization contained 100 μg of C5 protein in an incomplete Freund's adjuvant emulsion, and the third immunization was 100 μg of protein in PBS. The mice were injected at roughly 2 month intervals.

Fusions of splenocytes to myeloma cells to generate hybridomas were performed essentially as described in Current Protocols in Immunology (John Wiley & Sons, New York, 1992, pages 2.5.1 to 2.5.17). One day prior to fusion the mice were boosted IV with 100 μg of C5 protein. On the day of fusion, the immunized mice were sacrificed and spleens was harvested. SP2/0-AG14 myeloma cells (ATCC CRL#1581) were used as the fusion partner. SP2/0-AG14 cultures were split on the day before the fusion to induce active cell division. A ratio of 1:10 (myeloma cells:splenocytes) was used in the fusions.

The cells were fused using PEG 1450 in PBS without calcium (Sigma Chemical Company, St. Louis, Mo., Catalog No. P-7181) and plated at 1–2.5×10^5 cells per well. Selection in EX-CELL 300 medium (JRH Biosciences, Lexena, Kans., Catalog No. 14337-78P) supplemented with 10% heat inactivated fetal bovine serum (FBS); glutamine, penicillin and streptomycin (GPS); and HAT (Sigma Chemical Company, St. Louis, Mo., Catalog No. H-0262) was started the following day. The fusions were then fed every other day with fresh FBS, GPS, and HAT supplemented medium. Cell death could be seen as early as 2 days and viable cell clusters could be seen as early as 5 days after initiating selection. After two weeks of selection in HAT, surviving hybridomas chosen for further study were transferred to EX-CELL 300 medium supplemented with FBS, GPS, and HT (Sigma Chemical Company, St. Louis, Mo., Catalog No. H-0137) for 1 week and then cultured in EX-CELL 300 medium supplemented with FBS and GPS.

Hybridomas were screened for reactivity to C5 and inhibition of complement-mediated hemolysis 10–14 days after fusion, and were carried at least until the screening results were analyzed. The screen for inhibition of hemolysis was the chicken erythrocyte lysis assay described above. The screen for C5 reactivity was an ELISA, which was carried out using the following protocol:

A 50 μL aliquot of a 2 μg/mL solution of C5 (Quidel Corporation, San Diego, Calif.) in sodium carbonate /bicarbonate buffer, pH 9.5, was incubated overnight at 4° C. in each test well of a 96 well plate (NUNC-IMMUNO F96 POLYSORP, A/S Nunc, Roskilde, Denmark). The wells were then subjected to a wash step. (Each wash step consisted of three washes with TBST.) Next, test wells were blocked with 200 μL of blocking solution, 1% BSA in TBS (BSA/TBS) for 1 hour at 37° C. After an additional wash step, a 50 μL aliquot of hybridoma supernatant was incubated in each test well for 1 hour at 37° C. with a subsequent wash step. As a secondary (detection) antibody, 50 μL of a 1:2000 dilution of horseradish peroxidase (HRP) conjugated goat anti-mouse IgG in BSA/TBS, was incubated in each test well for 1 hour at 37° C., followed by a wash step. Following the manufacturer's procedures, 10 mg of O-phenylenediamine (Sigma Chemical Company, St. Louis, Mo., Catalog No. P-8287) was dissolved in 25 mLs of phosphate-citrate buffer (Sigma Chemical Company, St. Louis, Mo., Catalog No. P-4922), and 50 μL of this substrate solution was added to each well to allow detection of peroxidase activity. Finally, to stop the peroxidase detection reaction, a 50 μL aliquot of 3N hydrochloric acid was added to each well. The presence of antibodies reactive with C5 in the hybridoma supernatants was read out by a spectrophotometric OD determination at 490 nm.

The supernatant from a hybridoma designated as 5G1.1 tested positive by ELISA and substantially reduced the cell-lysing ability of complement present in normal human blood in the chicken erythrocyte hemolysis assay. Further analyses revealed that the 5G1.1 antibody reduces the cell-lysing ability of complement present in normal human blood so efficiently that, even when present at roughly one-half the molar concentration of human C5 in the hemolytic assay, it can almost completely neutralize serum hemolytic activity.

Immunoblot analysis was undertaken to further characterize the 5G1.1 mAb. Human C5 (Quidel Corporation, San Diego, Calif., Catalog No. A403) was subjected to polyacrylamide gel electrophoresis under reducing conditions, transferred to a nitrocellulose membrane, and probed with the 5G1.1 mAb as a purified IgG preparation. Two bands were immunoreactive with the 5G1.1 mAb at apparent molecular weights corresponding to those of the alpha and beta chains of the human C5 protein. The two 5G1.1 immunoreactive bands seen on this Western blot were subsequently found to result from the binding of the 5G1.1 antibody to the 115 kDa C5 alpha chain and to a large fragment of the alpha chain that had the same apparent molecular weight (approximately 75 kDa) as the beta chain of C5 and was present in the C5 preparations used for the experiment.

Assays were performed to determine the relative activity of the N19/8 mAb discussed in Examples 4 and 5 with the 5G1.1 mAb in functional hemolytic assays and to assess whether these mAbs blocked the cleavage of C5 to yield C5a. To this end, the N19/8 and 5G1.1 mAbs were directly compared in human complement hemolytic and C5a release assays.

Hemolytic assays performed in the presence of 20% v/v human serum revealed that the 5G1.1 mAb effectively blocked serum hemolytic activity at a final concentration of 6.25 μg/ml (0.5/1 molar ratio of 5G1.1/C5) whereas the N19/8 mAb blocked at a higher concentration of 25.0 μg/ml (2.0/1 molar ratio of N19/8/C5). When the supernatants from these assays were tested for the presence of C5a, the 5G1.1 mAb was found to have effectively inhibited C5a generation at doses identical to those required for the blockade of C5b-9 mediated hemolytic activity.

In contrast, the N19/8 mAb was 10 fold less effective in blocking the release of C5a in these assays when compared to the 5G1.1 mAb. Furthermore, the ability of the N19/8 mAb to block complement mediated hemolysis was not equivalent to its capacity to block C5a generation in that a dose of 25 μg/ml of N19/8 completely blocked hemolysis while only reducing C5a generation by 37%.

Hybridoma 5G1.1 was deposited with the American Type Culture Collection, 10801 University Boulevard, Manassas, Va. 20110-2209, United States of America, on Apr. 27, 1994, and has been assigned the designation HB-11625. This deposit were made under the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the Purposes of Patent Procedure (1977).

Example 8

Determination of the Affinity Constants (K_D) for the Anti-human C5 Monoclonal Antibodies 5G1.1 and N19/8

The procedure utilized to determine the dissociation constant (K_D) of antibody-antigen equilibria in solution was that described by Friguet et al., J. Immunol. Meth. 1985, 77:305–319. This method was used to determine the K_D for the anti-human C5 monoclonal antibodies N19/8 and 5G1.1. The monoclonal antibodies were incubated with the antigen (C5) in solution until the equilibrium was reached. The proportion of antibody that remains unbound (free) at equilibrium was measured using a conventional Enzyme Linked Immunosorbant Assay (ELISA). The experimental values of K_D obtained by this method have been shown to be equivalent to those obtained by other methods (immunoprecipitation of the radiolabeled antigen and fluorescence transfer). This method offers the advantage of dealing with unmodified antigen.

FIGS. **8** and **9** show the Scatchard plots of the binding of the anti-human C5 monoclonal antibodies 5G1.1 and N19/8 to human C5 as measured by ELISA. In each graph (v) represents the fraction of bound antibody and (a) represents the concentration of free antigen at equilibrium. The calculated K_D for the 5G1.1 mAb was 30 pM while the calculated K_D for the N19/8 mAb was 43 pM. These results indicate that the K_D for the 5G1.1 and N19/8 mab's are similar, and therefore the functional disparity between the two antibodies cannot be explained simply by the differences in affinity for the C5 antigen.

Example 9

Effect of 5G1.1 mAb on Complement Activation During CPB

Experiments involving recirculation of human blood in an CPB circuit, as described above in Examples 4 and 5, were carried out using three doses of the 5G1.1 mAb (15 mg, 7.5 mg, 3.75 mg) as well as controls in the absence of the 5G1.1 mAb. In five such control experiments performed in this series, C3a FIG. **10**) and sC5b-9 (FIG. **11**) levels increased during the first 30 min and continued to rise throughout the entire experiment. Addition of the 5G1.1 mAb to the CPB circuit had no effect on the generation of C3a in these experiments.

Conversely, addition of the two highest doses (15 mg and 7.5 mg) of the 5G1.1 mab completely blocked the generation of sC5b-9 in these experiments while the lowest dose (3.75 mg) only partially blocked sC5b-9 generation. Hemolytic assays performed on serum samples drawn throughout the time course of these experiments revealed that total serum complement activity was not affected in control experiments (FIG. **12**). In contrast, the highest dose of the 5G1.1 mAb (15 mg) completely blocked complement hemolytic activity, while the two lower doses (7.5 mg and 3.75 mg), failed to block hemolytic activity.

These results show that the 7.5 mg dose effectively blocked C5b-9 generation in the CPB circuit but failed to block C5b-9-mediated hemolytic activity, suggesting that hemolytic assays alone may not accurately reflect the complement activation that occurs during CPB. These results further indicate that the 5G1.1 mAb can completely block complement activation in human blood, as measured by either criterion, at a dosage of 15 mg/500 ml, a dose that is approximately equivalent to a dose of 150 mg for a 70 kg patient.

Example 10

Cloning of Anti-C5 Recombinant Anti-KSSKC Variable Region Genes Amino Acid Sequencing

To determine the N-terminal amino acid sequence of the 5G1.1 mAb, a 12% acrylamide gel (37.5:1 acrylamide/N, N'-methylene-bisacrylamide) was prepared and pre-electrophoresed for 45 minutes at 10 mA using 1× pre-electrophoresis buffer (123 mM bis-Tris, pH 6.6, with the cathode buffer reservoir supplemented with 1 mM reduced glutathione). The following day, the pre-electrophoresis buffer in the cathode reservoir was replaced with cathode reservoir buffer (44 mM N-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid, 113 mM bis-Tris, 0.1% (w/v) sodium dodecyl sulfate (SDS), 0.067% (w/v) thioglycolic acid) and the pre-electrophoresis buffer in the anode reservoir was replaced with anode reservoir buffer (63 mM bis-Tris, pH 5.9).

75 μg 5G1.1 monoclonal antibody was added to Laemmli sample buffer (30 mM Tris-HCl pH 6.8, 3% (w/v) SDS, 10 mM EDTA, 0.02% (w/v) bromophenol blue, 5% (v/v) glycerol, 2.5% (v/v) beta-mercaptoethanol) and electrophoresed at 10 mA until the bromophenol blue tracking dye reached the bottom of the gel. The protein was transferred to a PROBLOTT membrane (Applied Biosystems, Foster City, Calif.) using 1× transfer buffer (10 mM cyclohexylaminopropane sulfonic acid, 0.05% (w/v) dithiothreitol, 15% (v/v) methanol) at 50 V for one hour.

Protein bands were localized by staining with 0.2% Ponceau S (in 3% trichloroacetic acid, 3% sulfosalicylic acid) followed by destaining with water. Bands were excised and subjected to amino acid sequence analysis using Edman chemistry performed on a pulsed liquid protein sequencer (ABI model 477A), with the PTH amino acids thereby obtained being analyzed with an on-line microbore HPLC system (ABI model 120A).

To deblock the amino terminus of the 5G1.1 heavy chain, 10 mg 5G1.1 monoclonal antibody was exchanged into reducing buffer (5 M guanidine-HCl, 50 mM Tris-HCl, 10 mM dithiothreitol, pH 8.5) using a PD-10 column (Pharmacia, Piscataway, N.J.). After a one hour incubation at room temperature, 50 mM iodoacetamide was added and the incubation allowed to continue for 30 minutes. The carbamidomethylated light and heavy chains thus obtained were separated by size exclusion chromatography on a SUPEROSE 12 (Pharmacia) column equilibrated with 5 M guanidine-HCl, 50 mM Tris-HCl pH 8.5. The carbamidomethylated heavy chain was exchanged into 50 mM sodium phosphate, pH 7.0 using a PD-10 column, subjected to digestion with pyroglutamate aminopeptidase (PanVera, Madison, Wis.; 0.5 mU per nmol of heavy chain protein), and sequenced as described above.

For determination of internal amino acid sequence, the carbamidomethylated 5G1.1 light chain was exchanged into 2 M urea, 25 mM Tris-HCl, 1 mM EDTA, pH 8.0 and incubated with endoproteinase Lys-C (Promega, Madison, Wis.; protease:protein ratio of 1:40) at 37° C. overnight. The digested material was run on a C18 reversed phase HPLC column (Beckman Instruments, Fullerton, Calif.) and eluted using a linear 0–50% acetonitrile gradient in 0.1% trifluoroacetic acid. Peaks were subjected to amino acid sequence analysis as described above.

PCR Cloning

Cloning of the 5G1.1 variable heavy region was performed using a set of commercially available primers (Mouse Ig-PRIMER SET, catalogue number 69831-1, Novagen, Madison, Wis.). Total RNA was isolated from 5G1.1 hybridoma cells using the acid/guanidinium thiocyanate technique (Chomczynski and Sacchi, Anal. Biochem. 1987, 162:156–159). For first strand cDNA synthesis, ten micrograms total RNA were denatured at 65° C. for 5 min., chilled on ice, and added to a 100 μl reaction containing 10 mM Tris pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM dithiothreitol, 250 μM each dNTP, 20 units AMV reverse transcriptase (Seikagaku America, Rockville, Md.), and 10 pmole of the appropriate 3' primer (as described in the Ig-PRIMER SET kit protocol). After incubation at 37° C. for one hour, five microliters of the cDNA synthesis reaction were added to a 100 microliter PCR reaction containing: 10 mM Tris-HCl pH 9.0 at 25° C., 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% (w/v) gelatin, 1.0% (v/v) Triton X-100, 200 μM each dNTP, 2.5 U AMPLITAQ DNA polymerase (Perkin-Elmer-Cetus, Norwalk, Conn.) and 25 pmoles of the appropriate 5' and 3' primers (as described in the Ig-PRIMER SET kit protocol). The reaction conditions were 1 minute at 95° C., 1 minute at 42° C., and 1 minute at 72° C. for 30 cycles, followed by a final extension at 72° C. for 10 minutes.

PCR products having the expected size (approximately 450 bp) were cloned into the vector PCRII (Invitrogen, San Diego, Calif.) using a T/A cloning kit (Invitrogen). DNA sequence analysis of cloned DNA fragments was performed by the dideoxy chain-termination method using double-stranded plasmid DNA as a template. A unique heavy chain variable region was isolated by this procedure, with the resulting plasmid designated p5G1.1 VH 2-1-3. Several clones obtained from independent replicate PCR reactions were sequenced to detect any mutations introduced during the PCR amplification of this variable region.

To clone the 5G1.1 light chain variable region, PCR primers were designed by using the UWGCG program TFASTA (University of Wisconsin, Madison, Wis.) to search the GenBank rodent subdirectory with the 19mer query amino acid sequence Ile Gln Met Thr Gln Ser Pro Ala Ser Leu Ser Ala Ser Val Gly Glu Thr Val Thr, that was obtained by amino acid sequencing as described above. An exact match to this sequence was located in the murine germline gene encoding the v-kappa k2 variable region (Seidman et al. Proc. Natl. Acad. Sci. USA 1978 75:3881–3885). The DNA sequence of this germline gene was used to design the oligonucleotide UDEC690 (SEQ ID NO:5) for use as a variable region 5'-primer. A murine kappa gene constant region primer, UDEC395 (SEQ ID NO:6) was also synthesized and used in this reaction. Cloning of the 5G1.1 variable light region was performed using the UDEC690 variable region 5'-primer and the UDEC395 murine kappa gene constant region primer.

PolyA mRNA was isolated from hybridoma 5G1.1. The acid/guanidinium thiocyanate procedure (Chomczynski and Sacchi, supra) was used to isolate total RNA, and was followed by oligo(dT)-cellulose chromatography of 1 mg of total RNA. For first strand cDNA synthesis, one microliter of the 25 microliters of oligo(dT)-cellulose eluate (containing approximately 2 micrograms of purified 5G1.1 mRNA) was denatured at 65° C. for 5 min., chilled on ice, and incubated in extension buffer (10 mM Tris pH 8.3, 50 mM KCl, 1 mM dithithreitol, 240 μM each dNTP) containing 100 nM UDEC395 (SEQ ID NO:6) and 25 units AMV reverse transcriptase (Seikagaku America, Rockville, Md.) at 42° C. for one hour. Five microliters of the completed first strand reaction was subjected to PCR amplification using amplification buffer supplemented with 2.5 units AMPLITAQ DNA polymerase (Perkin Elmer, Foster City, Calif.) and 500 nM each of primer UDEC690 (SEQ ID NO:5) and UDEC395 (SEQ ID NO:6). Amplification was performed using 30 cycles each consisting of 1 minute at 95° C., 1 minute at 52° C., and 1 minute at 72° C., followed by a single ten minute incubation at 72° C.

The resulting PCR product was purified using GENECLEAN according to the manufacturer's directions (Bio 101, La Jolla, Calif.), digested with Sse8387 I and Hind III, gel purified, and ligated into the vector Bluescript II SK^+ (Stratagene, La Jolla, Calif.). Ligated plasmids were transformed into the bacterial strain DH10B by electroporation.

Plasmid DNA was purified from cultures of transformed bacteria by conventional methods including column chromatography using a QUIAGEN-TIP-500 column according to the manufacturer's directions (Quiagen, Chatsworth, Calif.) and sequenced by the Sanger dideoxy chain termination method using SEQUENASE enzyme (U.S. Biochemical, Cleveland, Ohio). Clones obtained from a second independent PCR reaction verified that no mutations were introduced during the amplification process. The resulting plasmid containing the cloned variable region was designated SK (+) 690/395. This light chain encoding insert in this plasmid coded for both the N-terminal and internal light chain sequences determined by amino acid sequencing of 5G1.1, as described above.

Example 11

Construction and Expression of Recombinant mAbs

Recombinant DNA constructions encoding the recombinant mAbs comprising the 5G1.1 CDRs are prepared by conventional recombinant DNA methods including restriction fragment subcloning and overlapping PCR procedures. The resulting recombinant mAb-encoding DNAs include:

(1) one encoding a non-humanized (murine) scFv designated 5G1.1M1scFv (SEQ ID NO:7), wherein CDR L1 is amino acid residues 28–34 of SEQ ID NO:7, CDR L2 is amino acid residues 52–54 of SEQ ID NO:7,

CDR L3 is amino acid residues 93–98 of SEQ ID NO:7, CDR H1 is amino acid residues 156–159 of SEQ ID NO:7, CDR H2 is amino acid residues 179–183 of SEQ ID NO:7, and CDR H3 is amino acid residues 226–236 of SEQ ID NO:7;

(2) one encoding a humanized (CDR grafted) scFv designated 5G1.1 scFv CB (SEQ ID NO:8), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:8, CDR L2 is amino acid residues 52–58 of SEQ ID NO:8, CDR L3 is amino acid residues 91–99 of SEQ ID NO:8, CDR H1 is amino acid residues 152–161 of SEQ ID NO:8, CDR H2 is amino acid residues 176–192 of SEQ ID NO:8, H3 is amino acid residues 225–237 of SEQ ID NO:8;

(3) one encoding a chimeric light chain (which can form the light chain portion of an Fab) designated 5G1.1M1 VL HuK (SEQ ID NO:9);

(4) one encoding a chimeric Fd (the heavy chain portion of an Fab) designated 5G1.1M1 VH HuG1 (SEQ ID NO:10);

(5) one encoding a humanized (CDR grafted and framework sequence altered) Fd designated 5G1.1 VH+IGHRL (SEQ ID NO:11), wherein CDR H1 is amino acid residues 26–35 of SEQ ID NO:11, CDR H2 is amino acid residues 50–60 of SEQ ID NO:11, and CDR H3 is amino acid residues 99–111 of SEQ ID NO:11;

(6) one encoding a humanized (CDR grafted, not framework altered) Fd designated 5G1.1 VH+IGHRLC (SEQ ID NO:12), CDR H1 is amino acid residues 26–35 of SEQ ID NO:12, CDR H2 is amino acid residues 50–66 of SEQ ID NO:12, and CDR H3 is amino acid residues 99–111 of SEQ ID NO:12;

(7) one encoding a humanized (CDR grafted and framework sequence altered) light chain designated 5G1.1 VL+KLV56 (SEQ ID NO:13), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:13, CDR L2 is amino acid residues 52–58 of SEQ ID NO:13, and CDR L3 is amino acid residues 91–99 of SEQ ID NO:13;

(8) one encoding a humanized (CDR grafted, not framework altered) light chain designated 5G1.1 VL+KLV56B (SEQ ID NO:14), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:14, CDR L2 is amino acid residues 52–58 of SEQ ID NO:14, and CDR L3 is amino acid residues 91–99 of SEQ ID NO:14;

(9) one encoding a humanized (CDR grafted, not framework altered) light chain designated 5G1.1 VL+012 (SEQ ID NO:15), wherein CDR L1 is amino acid residues 24–34 of SEQ ID NO:15, CDR L2 is amino acid residues 50–56 of SEQ ID NO:15, and CDR L3 is amino acid residues 89–97 of SEQ ID NO:15; and

(10) one encoding a humanized (CDR grafted, not framework altered) Fd designated 5G1.1 VH+IGHRLD (SEQ ID NO:16), wherein CDR H1 is amino acid residues 26–35 of SEQ ID NO:16, CDR H2 is amino acid residues 50–60 of SEQ ID NO:16, and CDR H3 is amino acid residues 99–111 of SEQ ID NO:16.

(11) one encoding a humanized (CDR grafted) scFv designated 5G1.1 scFv DO12 (SEQ ID NO:17), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:17, CDR L2 is amino acid residues 52–58 of SEQ ID NO:17, CDR L3 is amino acid residues 91–99 of SEQ ID NO:17, CDR H1 is amino acid residues 152–161 of SEQ ID NO:17, CDR H2 is amino acid residues 176–186 of SEQ ID NO:17, and CDR H3 is amino acid residues 225–237 of SEQ ID NO:17;

(12) one encoding a humanized (CDR grafted and framework sequence altered) scFv designated 5G1.1 scFv CO12 (SEQ ID NO:20), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:20, CDR L2 is amino acid residues 52–58 of SEQ ID NO:20, CDR L3 is amino acid residues 91–99 of SEQ ID NO:20, CDR H1 is amino acid residues 152–161 of SEQ ID NO:20, CDR H2 is amino acid residues 176–192 of SEQ ID NO:20, H3 is amino acid residues 225–237 of SEQ ID NO:20;

(13) one encoding a humanized (CDR grafted) scFv designated 5G1.1 scFv DO12B (SEQ ID NO:21), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:21, CDR L2 is amino acid residues 52–58 of SEQ ID NO:21, CDR L3 is amino acid residues 91–99 of SEQ ID NO:21, CDR H1 is amino acid residues 152–161 of SEQ ID NO:21, CDR H2 is amino acid residues 176–192 of SEQ ID NO:21, H3 is amino acid residues 225–237 of SEQ ID NO:21;

(14) one encoding a humanized (CDR grafted) scFv designated 5G1.1 scFv DO12C (SEQ ID NO:22), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:22, CDR L2 is amino acid residues 52–58 of SEQ ID NO:22, CDR L3 is amino acid residues 91–99 of SEQ ID NO:22, CDR H1 is amino acid residues 152–161 of SEQ ID NO:22, CDR H2 is amino acid residues 176–192 of SEQ ID NO:22, H3 is amino acid residues 225–237 of SEQ ID NO:22;

(15) one encoding a humanized (CDR grafted) scFv designated 5G1.1 scFv DO12D (SEQ ID NO:23), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:23, CDR L2 is amino acid residues 52–58 of SEQ ID NO:23, CDR L3 is amino acid residues 91–99 of SEQ ID NO:23, CDR H1 is amino acid residues 152–161 of SEQ ID NO:23, CDR H2 is amino acid residues 176–192 of SEQ ID NO:23, H3 is amino acid residues 225–237 of SEQ ID NO:23;

(16) one encoding a humanized (CDR grafted and framework sequence altered) scFv designated 5G1.1 scFv CO13 (SEQ ID NO:24), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:24, CDR L2 is amino acid residues 52–58 of SEQ ID NO:24, CDR L3 is amino acid residues 91–99 of SEQ ID NO:24, CDR H1 is amino acid residues 152–161 of SEQ ID NO:24, CDR H2 is amino acid residues 176–192 of SEQ ID NO:24, H3 is amino acid residues 225–237 of SEQ ID NO:24;

(17) one encoding a humanized (CDR grafted and framework sequence altered) scFv designated 5G1.1 scFv CO14 (SEQ ID NO:25), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:25, CDR L2 is amino acid residues 52–58 of SEQ ID NO:25, CDR L3 is amino acid residues 91–99 of SEQ ID NO:25, CDR H1 is amino acid residues 152–161 of SEQ ID NO:25, CDR H2 is amino acid residues 176–192 of SEQ ID NO:25, H3 is amino acid residues 225–237 of SEQ ID NO:25;

(18) one encoding a humanized (CDR grafted and framework sequence altered) scFv designated 5G1.1 scFv CO15 (SEQ ID NO:26), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:26, CDR L2 is amino acid residues 52–58 of SEQ ID NO:26, CDR L3 is amino acid residues 91–99 of SEQ ID NO:26, CDR H1

is amino acid residues 152–161 of SEQ ID NO:26, CDR H2 is amino acid residues 176–192 of SEQ ID NO:26, H3 is amino acid residues 225–237 of SEQ ID NO:26;

In accordance with the invention, one each of the various L1, L2 and L3 CDRs discussed in (1) to (18) above may be combined with any of the other light chain CDRs so as to make a set of 3 light chain CDRs comprising one L1, one L2, and one L3 CDR, as part of a recombinant antibody or synthetic peptide antibody (i.e., a synthetic peptide with the sequence of a recombinant peptide of the invention). Furthermore, the framework regions (i.e., the regions not included in the CDRs as described for each) of each of (1) to (18) above may be interchanged with homologous framework regions of the other recombinant antibody molecules of (1) to (18) to produce other antibodies of the invention.

In accordance with the invention, one each of the various H1, H2 and H3 CDRs discussed in (1) to (18) above may be combined with any of the other light chain CDRs so as to make a set of 3 light chain CDRs comprising one H1, one H2, and one H3 CDR, as part of a recombinant antibody or synthetic peptide antibody (i.e., a synthetic peptide with the sequence of a recombinant peptide of the invention).

In accordance with the invention, matched pairs of the variable regions (e.g., a VL and a VH region) of the various antibody molecules, Fds, and light chains described above may be combined with constant region domains by recombinant DNA or other methods known in the art to form full length antibodies of the invention. Particularly preferred constant regions for this purpose are IgG constant regions, which may be unaltered, or constructed of a mixture of constant domains from IgGs of various subtypes, e.g., IgG1 and IgG 4.

Matched pairs of the Fd and light chain encoding DNAs described immediately above—i.e. (3) and (4), (5) and (7), (6) and (8), and (6) and (9)—were subcloned together into the APEX-3P vector, essentially as described below in Example 15 for N19/8. The scFv constructs of (1) and (2) were subcloned into pET Trc SO5/NI using conventional techniques.

Plasmids so obtained were introduced by into the bacterial strain ME2 (pET plasmids) by conventional electroporation, or into human 293 EBNA cells (APEX plasmids) by lipofection using 2–3 microliters of TRANSFECTAM reagent (Promega, Madison, Wis.) per microgram of DNA according to the manufacturer's directions. Bacterial strains ME1 and ME2 are derivatives of *Escherichia coli* strain W3110 (ATCC designation 27325) prepared as follows.

Preparation of W3110 Derivatives ME1 and ME2

The non-humanized, non-chimeric murine 5G1.1-scFv "m5G1.1-scFv"—made up of light chain (3) and Fd (4)—was expressed in a derivative of *E. coli* K12 strain W3110. This derivative was prepared by inactivating an uncharacterized gene to provide protection against infections by a lytic bacteriophage. *E. coli* strain W3110 is a particularly preferred strain because it is fully characterized and is commonly used for recombinant DNA product fermentations.

A single colony of *E. coli* strain W3110 was grown overnight in L medium at 30° C. The cells were collected by centrifugation and resuspended in 10 mM $MgSO_4$. A total of 0.1 ml of the culture was added to 2.5 ml 0.7% L soft agar at 45° C. and quickly poured on an L plate. Fifty microliter aliquots of a plaque purified phage lysate, undiluted, diluted 10^{-2} and diluted 10^{-4}, were spotted onto the agar surface. Phage lysates had previously been filtered through 0.45 μm membranes and stored in sterile tubes with a drop of chloroform at 4° C. The spots were allowed to dry on the soft agar surface and incubated overnight at 37° C.

The next day L plates were spread with 10^9 phage PFU and allowed to dry. Using a sterile, flat toothpick, cells from isolated colonies growing in the zones of phage lysis on the spot plates were streaked for single colonies on the plates spread with 10^9 phage PFU and incubated overnight at 37° C. Single colonies were rechecked for phage resistance by cross-streaking after single colony purification. The cross streak test for phage sensitivity was performed as follows. Fifty μl of phage (10^8 pfu/ml) was spread in a vertical line in the left hand portion of the plate using a Pasteur pipette. Additional phage were tested parallel to the first and to the right. The plate was allowed to dry, and strains to be checked for sensitivity or resistance were spread perpendicular to and across the lines of all phages in a single swath from the left to the right. Resistant strains grow in the area of the phage streaks while sensitive strains lyse.

The phage resistant mutant strain ME1 was tested for phage production after overnight growth in L medium and treatment with the DNA damaging agent, mitomycin C. The strain failed to produce viable phage utilizing a standard plaque assay and *E. coli* W3110 as the phage sensitive indicator strain. These results suggest that strain ME1 does not harbor a resident prophage.

Strain ME2 was constructed by site specific integration of the lambdaDE3 prophage (Studier et al. 1990, Meth. Enzymol. 185:60–89) into the ME1 chromosome. Expression of the T7 RNA polymerase, directed by the prophage, allows expression of target genes cloned into pET vectors (Studier et al., supra) under the control of the T7 promoter in the lysogenized host. Lysogenization was accomplished in a three way infection with lambdaDE3, the lambda helper phage, lambdaB10 and the selection phage, lambdaB482 (Studier et al., supra).

lambdaDE3 (imm2l) was constructed by Studier and colleagues (1990, Meth. Enzymol. 185:60–89) by inserting the T7 RNA polymerase gene behind the *E. coli* lacUV5 promoter into the BamHI cloning site of lambdaD69 (imm21). Since cloning into the BamHI site of lambdaD69 interrupts the integrase gene, lambdaDE3 cannot integrate or excise from the chromosome by itself. The helper phage lambdaB10 provides the integrase function that lambdaDE3 lacks but cannot form a lysogen by itself. The selection phage, lambdaB482, lyses any lambdaDE3 host range mutants that otherwise would be among the surviving cells, but it can neither integrate into susceptible cells nor lyse lambdaDE3 lysogens since it has the same immunity region as lambdaDE3 (imm21).

Lysoaenization Protocol

Strain ME1 was grown in L medium supplemented with 0.2% maltose and 10 MM $MgSO_4$ at 37° C. to a density of approximately 10^8 cells/ml. One μl of ME1 cells were incubated with 2×10^8 plaque forming units (pfu) of lambdaDE3 and 10^8 pfu of lambdaB10 and lambdaB482. The host/phage mixture was incubated at 37° C. for 20 min to allow phage adsorption to ME1 cells. Several dilutions of the cell/phage suspension were spread on L plates to produce plates containing approximately 30–200 candidate lysogens as isolated colonies. The plates were inverted and incubated at 37° C. overnight. Several isolated colonies were screened for the acquisition of the lambdaDE3 prophage as described below.

Verification of lambdaDE3 Lysogens

lambdaDE3 lysogen candidates were tested for their ability to support the growth of the T7 phage 4107, a T7 phage deletion mutant that is completely defective unless active T7

RNA polymerase is provided in trans. Only lambdaDE3 lysogens will support the normal growth of the phage in the presence of IPTG (isopropyl-beta-thiogalactopyranoside). The T7 phage produces very large plaques on lambdaDE3 lysogens in the presence of IPTG, while very small plaques are observed in the absence of inducer. The size of the plaque in the absence of IPTG is an indication of the basal level of T7 RNA polymerase expression in the lysogen. Putative lambdaDE3 lysogens were grown in L broth supplemented with 0.2 % maltose and 10 mM $MgSO_4$ at 37° C. to a cell density of approximately 10^8 cells/ml. A total of 0.5 ml of cells was centrifuged and the pellet was resuspended in 0.2 ml of a T7 phage lysate containing 2×10^4 pfu. The phage was allowed to adsorb for 30 min at 37° C. One-half of suspension (0.1 ml) was added to 3.0 ml of molten top agarose at 47° C. and poured onto L plates. The remaining aliquot of cell/phage suspension was poured onto an L plate supplemented with 0.4 mM IPTG to check for induction of T7 RNA polymerase. The plates were inverted and incubated at 37° C. overnight.

Strains were also tested for the presence of the lambdaDE3 lysogen by demonstrating that each strain was resistant to infection by the phage lambdaB482, which is in the same immunity group (imm21), by the cross streak method described above. A lysogen was chosen with a low basal expression level for protein production from pET vectors. The resulting strain, designated ME2, is phage resistant and overexpresses T7 RNA polymerase in the presence of IPTG.

Purification of Humanized 5G1.1-scFv from *E. coli*

The humanized 5G1.1-scFv (h5G1.1-scFv) cDNA construct was cloned into the bacterial expression plasmid pET Trc SO5/NI (SEQ ID NO:18) and transformed into *E. coli* strain ME1. The resulting strain expressing h5G1.1 scFv was grown at 37° C. in 2 liter Applikon glass vessel fermentors containing Terrific Broth (1.2 % (w/v) bacto-tryptone, 2.4% (w/v) bacto-yeast extract, 0.4% (v/v) glycerol, 90 mM KPO_4, pH 7.0) supplemented with 100 μg/ml ampicillin. The production of recombinant scFv was induced by the addition of 1 mM IPTG when the $O.D._{\cdot550}$ of the culture reached 10. After an additional 3 h incubation at 37° C., the cells were harvested by centrifugation and the cell pellets stored at −80° C.

Cells were resuspended in 1 mM EDTA, pH 5.0 at 10 ml per gram weight and lysed by a single pass through a microfluidizer (Model M110T, Microfluidics Corp., Newton, Mass.). After centrifugation at 17,500×g for 15 min, the resulting inclusion body pellet was washed by resuspension in 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.15% (w/v) deoxycholate at 10 ml per gram inclusion body using a Tekmar POLYTRON. The inclusion bodies were again pelleted by centrifugation at 17,500×g for 15 min and resuspended in 20 mM Tris-HCl pH 9.0, 8 M urea at 10 ml per g. After stirring for 1 h, the sample was centrifuged at 14,000×g for 30 min to pellet remaining insoluble material.

The extract supernatant was diluted 10-fold with 20 mM Tris-HCL pH 9.0, 7 M urea, 50 μM cupric sulfate and allowed to stir for at least 16 hours at 4° C. to refold the scFv. After addition of BIOCRYL BPA-1000 (TosoHaas, Montgomeryville, Pa.) as a flocculating agent at 3 μl per ml, the sample was centrifuged at 15,000×g for 10 minutes to remove insoluble material. The refolding mixture was exchanged into 20 mM Tris, pH 9.0, 1 mM EDTA by diafiltration and concentrated by ultrafiltration using a stirred cell fitted with a YM10 membrane (Amicon, Beverly, Mass.).

In subsequent experiments, other refolding conditions were tested. Thawed bacterial cells were resuspended with a POLYTRON homogenizer in 1 mM EDTA at 2.5 mL per gram of cells, passed through the MICROFLUIDIZER at 18,000 psi, and centrifuged at 10,000 RPM for 15 min in a Beckman JA-10 rotor, the resulting pellet was washed by resuspension in 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.15% (w/v) deoxycholate at 10 ml per gram inclusion body using a Tekmar POLYTRON. The inclusion bodies were again pelleted by centrifugation. The pellet from this centrifugation was resuspended with a POLYTRON homogenizer in 8M urea, 20 mM Tris pH9 at 10 mL per gram of pellet. After stirring for 1 hour at 4 degrees C., the resuspended pellet was diluted with 9 volumes of 7M urea, 20 mM Tris pH9. Cupric sulfate was then added to various final concentrations (0, 5, 10, 20, 25, 30, 40, 50, 100, 150, and 200 μM) before incubation overnight at 4 degrees C. with stirring. The use of 5 μM copper was found to give the highest levels of refolding of the humanized 5G1.1-scFv as assessed by analytical HPLC.

In the initial experiments, the properly refolded scFv was then separated from aggregated material and contaminating proteins by anion exchange chromatography using Q SEPHAROSE FAST FLOW (Pharmacia, Piscataway, N.J.). Bound scFv was eluted with 20 mM Tris-HCL pH 9.0, 1 mM EDTA containing a linear NaCl gradient (0 to 0.5 M). The fractions containing the scFv were combined, concentrated by ultrafiltration using a stirred cell fitted with a YM10 membrane, and applied to a SEPHACRYL S200 HR 26/100 gel filtration column (Pharmacia) equilibrated in 20 mM Tris-HCL pH 9.0, 1 mM EDTA, 150 mM NaCl. Fractions containing the scFv were combined, exchanged into phosphate-buffered saline by diafiltration, concentrated by ultrafiltration, filtered through a 0.22 μm MILLEX-GV filter (Millipore, Bedford, Mass.), and stored at 4° C.

Subsequent experiments have indicated that cation exchange chromatography (e.g., using POROS HS resin—PerSeptive Biosystems, Cambridge, Mass.) should give better yields than the Q Sepharose Fast Flow anion exchange chromatography step described in the preceding paragraph. In addition, it would be preferable to carry out the final gel filtration chromatography in a buffer that is more pharmaceutically acceptable than the Tris buffer described. A buffer such as PBS would be preferred if it does not interfere with the efficacy of the gel filtration chromatographic separation. This would reduce any trace amounts of Tris remaining in the preparation after diafiltration, and might eliminate the need for the diafiltration step.

Purification of m5G1.1-scFv from *E. coli*

Frozen bacterial cell paste was thawed and resuspended in 2.5 ml of 1 MM EDTA (pH 5) per gram of cell paste. This suspension of cells was lysed by passage through a MICROFLUIDIZER (Microfluidics) with the interaction chamber in line and a backpressure of approximately 18000 psi. The cell lysate was then centrifuged at 10,000 rpm in a JA-10 centrifuge rotor at 4° C. for 15 min. The supernatant was decanted and discarded.

The pellet was resuspended in 10 ml of 20 mM Tris, pH 8.0, 100 mM NaCl, 0.15% sodium deoxycholate per gram of pellet. This suspension was centrifuged as above for 10 min. Again the supernatant was decanted and discarded. This detergent washed pellet was then resuspended in 10 ml of 8 M urea, 20 mM Tris-HCl, pH 9 (1 mM EDTA may also be added to this buffer, but has the effect of increasing the time required to achieve a particular level of refolding). The suspension was stirred at 4° C. for 1 hr. and was then diluted 10 fold with 7 M urea, 20 mM Tris-HCl, pH 9 and stirred at 4° C. $CUSO_4$ was then added to a final concentration of 50 μM and stirring was continued overnight at 4° C.

The majority of contaminating proteins (including incorrectly folded versions of m5G1.1 scFv) were then removed by precipitation by diluting (with stirring) the refolded sample five fold with buffer such that the final concentrations after dilution were 1.4 M urea, 25 mM NaCl, 1 mM EDTA, and 20 mM sodium acetate at 4° C. The pH of the dilution buffer when prepared at room temperature was pH 5.0. Prior to dilution the pH of the dilution buffer is determined at 4° C. After the dilution the pH of the sample was greater than pH 5.5. The pH of the sample was then adjusted with 6. N HCl to the initial pH 5.0 of the buffer at 4° C. The solution immediately became cloudy and it was left stirring at 4–8° C. for 0.5 to 24 hours.

The precipitate was removed by filtering the sample through a 300 kDa cut-off ultrafiltration membrane (Millipore Corporation, Bedford, Mass.). The permeate was collected and concentrated 5 fold using a 10 kDa cutoff ultrafiltration membrane (Millipore). This concentrated retentate was then diluted 2 fold with 20 mM sodium acetate, 1 mM EDTA, pH 5.0 in order to lower the NaCl concentration to 12.5 mM.

The diluted retentate was then loaded at 4° C. onto a SP SEPHAROSE FF column (Pharmacia) equilibrated in 0.7 M urea, 1 mM EDTA, 10 mM NaCl, 20 mM sodium acetate, pH 5.0, at a linear flowrate of 5 cm/min. Bed height was equal to or greater than 3.5 cm. Following loading the column was washed with 40 column volumes (CV) of equilibration buffer. The column was then washed with 20 CV of 20 mM sodium acetate, pH 5.0, 1 mM EDTA. The bound scFv was then eluted using 20 mM sodium citrate, pH 5.8, 1 mM EDTA. A single peak was collected in approximately 4 column volumes.

The SP SEPHAROSE eluate was then adjusted to 20 mM Tris-HCL by addition of 1 M Tris-HCL, pH 8. The pH of the sample was adjusted to 8.0 by addition of 1 N NaOH. This sample was loaded onto a Q SEPHAROSE FF column (Pharmacia) equilibrated in 20 mM Tris-HCL, pH 8.0, 1 mM EDTA at room temperature at a flowrate of 5 cm/min. The flow through fraction containing the scFv was collected.

The Q SEPHAROSE flow through fraction was then adjusted to 150 mM NaCl and concentrated to 10 mg of scFv per ml using a 10 kDa cutoff ultrafiltration membrane. This concentrated sample was then loaded onto a SEPHACRYL S200 column equilibrated in phosphate buffered saline, pH 7.4 and eluted at 0.4 cm/min. The fractions were analyzed by SDS-PAGE and silver staining. Peak fractions were combined after discarding the front and back shoulder fractions that contained the majority of contaminants.

Example 12

Functional Analysis of the m5G1.1 scFv

Titration of the m5G1.1 scFv in hemolytic assays revealed that the m5G1.1 scFv inhibited human complement-mediated lysis in a dose dependent fashion (FIG. **13**). Direct comparison of the efficacy of the m5G1.1 scFv to the 5G1.1 mAb and Fab demonstrated that the m5G1.1 scFv completely blocked C5b-9-mediated hemolysis in 20% human serum at 0.15 μM while the 5G1.1 mAb and Fab blocked at 0.06–0.08 μM. Analysis of C5a generation in these assays revealed similar results in that the 5G1.1 scfv completely blocked C5a generation at 0.15 μM while the 5G1.1 mAb and Fab blocked at 0.06–0.08 μM (FIG. **14**). Taken together these experiments indicated that unlike N19/8, which lost half of its effectiveness at blocking C5a generation upon being engineered as an scFv (SEQ ID NO:19), the 5G1.1 murine scFv retained the capacity to block the generation of both C5a and C5b-9.

Additionally, these data demonstrate that the m5G1.1 scFv retained similar activity to that of the parent molecule (the native murine 5G1.1 mAb) in that the molar concentration of 5G1.1 murine scFv required to completely block C5a and C5b-9 (0.15 μM) was within two-fold of that required for the 5G1.1 mAb and Fab (0.06–0.08 μM).

In order to determine whether the m5G1.1 scFv retained the capacity to block the activation of complement in the ex vivo model of cardiopulmonary bypass, 4.5 mg of the purified bacterially produced 5G1.1 murine scFv was added to the CPB circuit and complement activation was monitored. In control experiments, both C3a and C5b-9 levels increased throughout the time course of the experiment. In a single experiment, addition of 4.5 mg of the m5G1.1 scFv to the CPB circuit had no effect on the generation of C3a (FIG. **15**). Conversely, complement hemolytic activity as well as the generation of sC5b-9 was completely blocked in this experiment (FIG. **16** and FIG. **17**).

Example 13

Characterization of the Epitope Recognized by 5G1.1

Tryptic digestion: Twenty micrograms of purified human C5 (Advanced Technologies, San Diego, Calif.) was subjected to enzymatic digestion with 1 μg of TPCK-treated trypsin (Worthington Biochemical Corp., Freehold, N.J.). The digestion was allowed to continue for 3 minutes, after which time it was stopped by the addition of 20 μg soy bean trypsin inhibitor (Worthington). The reaction was then denatured and reduced by the addition of protein sample buffer and immediately boiled for 5 min. The digested fragments were size fractionated through a SDS-PAGE on a 12 % gel. The gel was then electroblotted in transfer buffer (20% (v/v) methanol, 25 mM Tris-base pH 8.0, and 192 mM glycine) to nitrocellulose (Bio-Rad Laboratories, Hercules, Calif.) and subjected to ECL western blot analysis using either 5G1.1 or a C5a specific monoclonal antibody (G25/2, obtained from Dr. Otto Götze, Department of Immunology, University of Göttingen, Germany).

The filters were incubated twice for 30 minutes each in blocking solution (500 mM NaCl, 5 mM Tris p-H 7.4, 10% (v/v) nonfat dry milk, and 0.2% (v/v) TWEEN-20). The filters were then changed to fresh blocking solution (20 ml) containing the primary antibody and incubated for 40 minutes on a rocking platform. The filters were rinsed briefly with washing solution (500 mM NaCl, 35 mM Tris pH7.4, 0.1% SDS, 1% NP40, and 0.5% deoxycholic acid) to remove any milk, and then fresh wash solution was added and incubated for two 20 minute intervals on an orbiting shaker. The filters were rinsed briefly with 10 to 20 mls of secondary antibody solution (500 mM NaCl, 5 mM Tris pH 7.4, 10% (v/v) Nonfat dry milk, 0.2% (v/v) TWEEN-20, and 1% NP-40) and then incubated with fresh secondary antibody solution containing a 1:2000 dilution of HRP conjugated goat anti-mouse for 20 minutes on a rocking platform. The filters were then washed as described above, incubated in ECL reagent (Amersham Corp., Arlington Heights, Ill.) for 1 minute and then exposed to ECL HYPERFILM (Amersham).

Acid Hydrolysis: Twenty micrograms of purified human C5 (Advanced Technologies) was subjected to hydrolysis in 1N acetic acid. The 20 μg of human C5 (1 μg/μl) was added to 20 μl of 2N acetic acid and incubated for 10 min at 100° C. The sample was denatured and reduced with protein sample buffer, also at 100° C., for 5 minutes. The acid was

neutralized by dropwise addition of a saturated tris base solution until the sample turned blue. The cleavage products were then size fractionated by SDS-PAGE and western blotted as described above. For N-terminal sequencing, the gel fractionated acid hydrolysate was transferred to PVDF membrane. N-terminal sequence was obtained by excising the 46 kDa acid hydrolysis fragment band from a PVDF membrane and subjecting it to amino acid sequence analysis as discussed above in Example 10.

Deglycosylation: Reduced and denatured acid hydrolyzed or tryptic fragments of human C5 were subjected to deglycosylation with N-Glycosidase F (Peptide-N-Glycosidase F, Boehringer Mannheim Corp., Indianapolis, Ind.) according to the manufacture's directions.

Results: Acid hydrolysis of human C5 yielded a fragment with an apparent molecular weight by SDS-PAGE of 46 kDa that was immunoreactive for both the anti-C5a mAb G25/2 and the anti-C5 alpha chain mAb 5G1.1. Western blots probed with both antibodies simultaneously, as well as silver stain SDS-PAGE analysis, confirmed the presence of a single 46 kDa fragment that was immunoreactive with both antibodies. The presence of a single immunoreactive fragment containing binding sites for both 5G1.1 and G25/2 strongly suggested that the 5G1.1 epitope was contained within approximately the first 46 kDa of the N-terminus of the alpha chain of C5.

As discussed above in the description of the complement system under the heading "Background Physiology & Pathology," a compound (e.g., an antibody) that binds to a site at or immediately adjacent to the C5a cleavage site would have the potential to act as a terminal complement inhibitor. The potential inhibitory activity of antibodies binding to this site led to the expectation that the C5 alpha chain-binding 5G1.1 antibody would bind to an epitope at or near the C5a cleavage site. The finding that 5G1.1 bound to the 46 kDa acid hydrolysis fragment of C5 lent support to this expectation.

Western blot analysis of the tryptic digestion products identified one proteolytic fragment migrating at approximately 27 kDa that was immunoreactive with 5G1.1. Likewise, one immunoreactive proteolytic fragment migrating at approximately 29 kDa was observed following western blot analysis with the anti-C5a mAb G25/2. Experiments in which a blot was simultaneously probed with both 5G1.1 and G25/2 demonstrated that each band was distinct and that their apparent differential mobility was not a gel anomaly. This was surprising, because the 5G1.1 mAb was thought likely to bind to the C5 convertase cleavage site. 5G1.1 was thus expected to be immunoreactive with any fragment of C5 of over 12 kDa that exhibited immunoreactivity with G25/2. Such a fragment would contain enough of the extreme amino terminus of the C5 alpha chain to bind specifically to the anti-C5a mAb, and enough beyond that to encompass a region including and extending beyond the C5 convertase cleavage site.

The immunoreactivity of G25/2 with the 29 kDa fragment indicated that that fragment contains the N-terminal region of the alpha chain of C5 that is cleaved off to yield C5a. Furthermore, because 5G1.1 was not immunoreactive with this band, the 5G1.1 epitope was not likely to be contained within approximately the first 29 kDa of the N-terminus of the alpha chain of C5, and therefore could not be located near the C5 convertase cleavage site.

These tryptic digestion and acid hydrolysis mapping data suggested that the 5G1.1 epitope was contained within a region starting about 29 kDa (including post-translational modifications) from the N-terminus of the alpha chain of C5 and continuing 17 kDa in a C-terminal direction, i.e., ending 46 kDa from the N-terminus, a surprising finding in view of the expectation, discussed above, that the antibody would bind at or immediately adjacent to the point at which C5a is cleaved off of the C5 alpha chain, i.e., at or immediately adjacent to amino acid residue 733 of SEQ ID NO:2.

Post-translational modifications can alter the mobility of proteins during SDS-PAGE electrophoresis. One such modification is the addition of carbohydrate via N-linked glycosylation. As discussed above under the heading "Background Physiology & Pathology", C5 is glycosylated, as is C5a. C5a is glycosylated at an asparagine residue corresponding to amino acid number 723 of the full length pro-C5 precursor of human C5 (SEQ ID NO:2).

Computer analysis of the human C5 alpha chain suggests potential N-linked glycosylation sites at positions corresponding to amino acid numbers 893, 1097, and 1612 of SEQ ID NO:2. In order to determine the contribution of carbohydrate to the electrophoretic mobility of both the tryptic and acid fragments, enzymatic deglycosylation of the fragments was performed and followed by western blot analysis. It was determined that each tryptic fragment lost approximately 3 kDa in apparent molecular weight while the acid fragment lost approximately 6 kDa.

This result was interpreted as indicating that the tryptic fragments were each glycosylated at a single site and that the 46 kDa acid fragment was glycosylated at two sites (one of which was the known glycosylation site in C5a referred to above). The diminished mobility observed following deglycosylation agrees with the computed prediction of a second N-linked glycosylation site within the first 233 amino acids of the C5 alpha chain.

N-terminal sequence analysis determined that the first four amino acids of the 46 kDa fragment generated by 1N acetic acid treatment was Thr Leu Gln Lys. This sequence is found only once in the full length human pro-C5 precursor molecule—at a position corresponding to amino acids 660 through 663 of SEQ ID NO:2. This four amino acid sequence also corresponds to the sequence of the amino-terminus of the alpha chain of human C5 and, thus to the amino-terminus of human C5a.

In order to more precisely map the binding site of 5G1.1, overlapping peptide analysis was performed. The sequence predicted to be contained within the 17 kDa section of human C5 described above (SEQ ID NO:2; amino acids 893 through 1019) together with an extension of 43 amino acids towards the N-terminus and 30 amino acids towards the C-terminus (a total of 200 amino acids) was synthesized as a series of 88 overlapping peptides by solid phase synthesis on polypropylene filters (Research Genetics Inc., Huntsville, Ala.).

The 43 and 30 amino acid extensions were added to allow for possible inaccuracies in the prediction of the span of this 17 kDa region. Such inaccuracies are likely due to the uncertainty of the specific extent of glycosylation of each of the various regions of C5a, as well as to the aberrant gel mobility that is commonly seen when highly charged polypeptides (such as the 5G46k fragment and the 5G27k fragment) are analyzed by SDS-PAGE. As discussed above in the Summary of the Invention, a 200 amino acid peptide corresponding to the region covered by these overlapping peptides is referred to herein as the "5G200aa" peptide.

Because of the expectation that the 5G1.1 antibody would bind at the C5a cleavage site, an additional set of 8 overlapping peptides was synthesized that spanned a 30 amino

acid section spanning the C5a cleavage site (amino acids 725 through 754 of SEQ ID NO:2). A peptide having the sequence of this 30 amino acid section is referred to herein as the "cleavage site peptide". A 325aa peptide spanning amino acid residues 725–1049 of SEQ ID NO:2 (this peptide spans the region covered by the cleavage site peptide and the 5G200aa peptide) is referred to herein as the "5G325aa" peptide.

These filters were probed with 5G1.1 as described above for ECL western blot analysis, and a set of 4 overlapping peptides spanning the region corresponding to amino acid residues 3–19 of the KSSKC peptide (SEQ ID NO:1) each gave a positive signal indicative of monoclonal antibody binding, while peptides corresponding to the C5a cleavage site did not bind to the 5G1.1 antibody.

Example 14

C3/C4 Binding Assay

C3 and C4 are both key components of classical C5 convertase, and C3 is also a key component of alternative C5 convertase. These C5 convertases are required for the conversion of C5 to active C5a and C5b. The ability to block C5 binding to C3 and C4 is thus a desirable property for an antibody to be used in treatment of complement mediated diseases in accordance with the present invention.

96 well microtiter plates were coated with 501 μ/well, 10 μg/ml of either purified human C3 or C4 (Quidel) for 1 hour at 37° C. The plates were then blocked with 200 μl/well of TBS containing 1% BSA for 1 hour at room temperature. After three washes in TBS 0.1% BSA, purified human C5 (Quidel, 20 μg/ml in TBS 1% BSA) was added to the plates in the presence (20 μg/ml) or absence of a 5G1.1 Fab (derived from 5G1.1 by conventional papain digestion) and allowed to incubate for 1 hour at 37° C. After three washes in TBS/0.1% BSA, a monoclonal antibody directed against the C5 beta chain (N19/8, 5 μg/ml) was added to the wells to detect C5 bound to either C3 or C4. After three final washes in TBS/0.1% BSA, the plate was developed using a horseradish peroxidase-conjugated secondary antibody and the appropriate substrate.

The results of these assays showed that the 5G1.1 mAb inhibited the binding of purified human C5 to either C3 or C4 by at least 60% to 90%. As used herein and in the claims, such a 60% to 90% reduction in C3 or C4 binding is a "substantial reduction" in C3 or C4 binding.

Example 15

Construction and Functional Analysis of N19/8 Chimeric Fab

The heavy chain and light chain variable regions from the hybridoma N19-8 were cloned by PCR using the Ig-Prime System (Novagen) as described by the manufacturer. Clones from multiple independent PCR reactions were sequenced to detect mutations introduced during the PCR amplification. An N19-8 VL/human kappa constant region chimeric cDNA was created by using a plasmid containing the N19-8 light chain variable region and the plasmid pHuCK (Hieter et al., 1980 Cell, 22:197–207) as templates in an overlapping PCR reaction.

Similarly, an N19-8 VH/human IgG1 Fd chimeric cDNA was created using a plasmid containing the N19-8 heavy chain variable region and a plasmid containing the human IgG1 gene (obtained from Ilan R. Kirsch, National Cancer Institute, Bethesda, Md.) as templates. This Fd construct contained the first nine amino acids of the IgG1 hinge region, including the cysteine residue which normally forms a disulfide bond with the terminal cysteine residue of the kappa light chain.

The resulting chimeric cDNAs were separately cloned into the APEX-1 vector using appropriate flanking restriction enzyme sites introduced during the PCR amplification procedure and sequenced. A fragment containing the promoter, intron, and cDNA insert from one of these APEX vectors was subsequently subcloned into the polylinker of the other to produce a single vector directing the expression of both the light chain and Fd. The tandem expression cassette from this APEX-1 vector was subsequently subcloned into APEX-3P, which was transfected into 293 EBNA cells for expression of the chimeric Fab.

When tested for the ability to block complement hemolytic activity and C5a generation, the chimeric N19/8 Fab retained the ability to block hemolytic activity, but lost 50% of its C5a generation blocking capacity.

Throughout this application various publications and patent disclosures are referred to. The teachings and disclosures thereof, in their entireties, are hereby incorporated by reference into this application to more fully describe the state of the art to which the present invention pertains.

Although preferred and other embodiments of the invention have been described herein, further embodiments may be perceived by those skilled in the art without departing from the scope of the invention as defined by the following claims.

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TABLE 1

Prevention/Reduction of Proteinuria by Treatment With Anti-C5 Antibodies

	Before Treatment Urine Protein (mg/dL)	After Treatment Urine Protein (mg/dL)
	PBS Control	
mouse A	none	100
mouse B	none	500
mouse C	none	500
mouse D*	trace	trace
mouse E	100	100
	Anti-C5 Treated	
mouse 1	none	none
mouse 2	none	30
mouse 3	30	trace
mouse 4	30	30
mouse 5	30	30
mouse 6	100	30

*Mouse D had more than 500 mg/dL urine glucose after treatment

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 26

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: Amino Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE:
(A) DESCRIPTION: KSSKC peptide

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Val Ile Asp His Gln Gly Thr Lys Ser Ser
5 10

Lys Cys Val Arg Gln Lys Val Glu Gly Ser Ser
15 20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1676 Amino Acids
(B) TYPE: Amino Acid

-continued

(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE:
(A) DESCRIPTION: Pro-C5 Polytpeptide

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Haviland, D.L.
Haviland, J.C.
Fleischer, D.T.
Hunt, A.
Wetsel, R.A.
(B) TITLE: Complete cDNA Sequence of Human
Complement Pro-C5
(C) JOURNAL: Journal of Immunology
(D) VOLUME: 146
(F) PAGES: 362-368
(G) DATE: 1991

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Gly Leu Leu Gly Ile Leu Cys Phe Leu
-15                 -10

Ile Phe Leu Gly Lys Thr Trp Gly Gln Glu Gln Thr Tyr Val
-5              -1                   5

Ile Ser Ala Pro Lys Ile Phe Arg Val Gly Ala Ser Glu Asn
10                  15                  20

Ile Val Ile Gln Val Tyr Gly Tyr Thr Glu Ala Phe Asp Ala
25                  30

Thr Ile Ser Ile Lys Ser Tyr Pro Asp Lys Lys Phe Ser Tyr
35                  40                  45

Ser Ser Gly His Val His Leu Ser Ser Glu Asn Lys Phe Gln
50                  55                  60

Asn Ser Ala Ile Leu Thr Ile Gln Pro Lys Gln Leu Pro Gly
65                  70                  75

Gly Gln Asn Pro Val Ser Tyr Val Tyr Leu Glu Val Val Ser
80                  85                  90

Lys His Phe Ser Lys Ser Lys Arg Met Pro Ile Thr Tyr Asp
95                 100

Asn Gly Phe Leu Phe Ile His Thr Asp Lys Pro Val Tyr Thr
105                 110                 115

Pro Asp Gln Ser Val Lys Val Arg Val Tyr Ser Leu Asn Asp
120                 125                 130

Asp Leu Lys Pro Ala Lys Arg Glu Thr Val Leu Thr Phe Ile
135                 140                 145

Asp Pro Glu Gly Ser Glu Val Asp Met Val Glu Glu Ile Asp
150                 155                 160

His Ile Gly Ile Ile Ser Phe Pro Asp Phe Lys Ile Pro Ser
165                 170

Asn Pro Arg Tyr Gly Met Trp Thr Ile Lys Ala Lys Tyr Lys
175                 180                 185

Glu Asp Phe Ser Thr Thr Gly Thr Ala Tyr Phe Glu Val Lys
190                 195                 200

Glu Tyr Val Leu Pro His Phe Ser Val Ser Ile Glu Pro Glu
205                 210                 215
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Tyr Asn Phe Ile Gly Tyr Lys Asn Phe Lys Asn Phe Glu Ile
220                 225                 230

Thr Ile Lys Ala Arg Tyr Phe Tyr Asn Lys Val Val Thr Glu
235                 240

Ala Asp Val Tyr Ile Thr Phe Gly Ile Arg Glu Asp Leu Lys
245                 250                 255

Asp Asp Gln Lys Glu Met Met Gln Thr Ala Met Gln Asn Thr
260                 265                 270

Met Leu Ile Asn Gly Ile Ala Gln Val Thr Phe Asp Ser Glu
275                 280                 285

Thr Ala Val Lys Glu Leu Ser Tyr Tyr Ser Leu Glu Asp Leu
290                 295                 300

Asn Asn Lys Tyr Leu Tyr Ile Ala Val Thr Val Ile Glu Ser
305                 310

Thr Gly Gly Phe Ser Glu Glu Ala Glu Ile Pro Gly Ile Lys
315                 320                 325

Tyr Val Leu Ser Pro Tyr Lys Leu Asn Leu Val Ala Thr Pro
330                 335                 340

Leu Phe Leu Lys Pro Gly Ile Pro Tyr Pro Ile Lys Val Gln
345                 350                 355

Val Lys Asp Ser Leu Asp Gln Leu Val Gly Gly Val Pro Val
360                 365                 370

Ile Leu Asn Ala Gln Thr Ile Asp Val Asn Gln Glu Thr Ser
375                 380

Asp Leu Asp Pro Ser Lys Ser Val Thr Arg Val Asp Asp Gly
385                 390                 395

Val Ala Ser Phe Val Leu Asn Leu Pro Ser Gly Val Thr Val
400                 405                 410

Leu Glu Phe Asn Val Lys Thr Asp Ala Pro Asp Leu Pro Glu
415                 420                 425

Glu Asn Gln Ala Arg Glu Gly Tyr Arg Ala Ile Ala Tyr Ser
430                 435                 440

Ser Leu Ser Gln Ser Tyr Leu Tyr Ile Asp Trp Thr Asp Asn
445                 450

His Lys Ala Leu Leu Val Gly Glu His Leu Asn Ile Ile Val
455                 460                 465

Thr Pro Lys Ser Pro Tyr Ile Asp Lys Ile Thr His Tyr Asn
470                 475                 480

Tyr Leu Ile Leu Ser Lys Gly Lys Ile Ile His Phe Gly Thr
485                 490                 495

Arg Glu Lys Phe Ser Asp Ala Ser Tyr Gln Ser Ile Asn Ile
500                 505                 510

Pro Val Thr Gln Asn Met Val Pro Ser Ser Arg Leu Leu Val
515                 520

Tyr Tyr Ile Val Thr Gly Glu Gln Thr Ala Glu Leu Val Ser
525                 530                 535

Asp Ser Val Trp Leu Asn Ile Glu Glu Lys Cys Gly Asn Gln
540                 545                 550

Leu Gln Val His Leu Ser Pro Asp Ala Asp Ala Tyr Ser Pro
555                 560                 565

Gly Gln Thr Val Ser Leu Asn Met Ala Thr Gly Met Asp Ser
570                 575                 580
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Trp Val Ala Leu Ala Ala Val Asp Ser Ala Val Tyr Gly Val
585                 590

Gln Arg Gly Ala Lys Lys Pro Leu Glu Arg Val Phe Gln Phe
595                 600                 605

Leu Glu Lys Ser Asp Leu Gly Cys Gly Ala Gly Gly Gly Leu
610                 615                 620

Asn Asn Ala Asn Val Phe His Leu Ala Gly Leu Thr Phe Leu
625                 630                 635

Thr Asn Ala Asn Ala Asp Asp Ser Gln Glu Asn Asp Glu Pro
640                 645                 650

Cys Lys Glu Ile Leu Arg Pro Arg Arg Thr Leu Gln Lys Lys
655                 660

Ile Glu Glu Ile Ala Ala Lys Tyr Lys His Ser Val Val Lys
665                 670                 675

Lys Cys Cys Tyr Asp Gly Ala Cys Val Asn Asn Asp Glu Thr
680                 685                 690

Cys Glu Gln Arg Ala Ala Arg Ile Ser Leu Gly Pro Arg Cys
695                 700                 705

Ile Lys Ala Phe Thr Glu Cys Cys Val Val Ala Ser Gln Leu
710                 715                 720

Arg Ala Asn Ile Ser His Lys Asp Met Gln Leu Gly Arg Leu
725                 730

His Met Lys Thr Leu Leu Pro Val Ser Lys Pro Glu Ile Arg
735                 740                 745

Ser Tyr Phe Pro Glu Ser Trp Leu Trp Glu Val His Leu Val
750                 755                 760

Pro Arg Arg Lys Gln Leu Gln Phe Ala Leu Pro Asp Ser Leu
765                 770                 775

Thr Thr Trp Glu Ile Gln Gly Ile Gly Ile Ser Asn Thr Gly
780                 785                 790

Ile Cys Val Ala Asp Thr Val Lys Ala Lys Val Phe Lys Asp
795                 800

Val Phe Leu Glu Met Asn Ile Pro Tyr Ser Val Val Arg Gly
805                 810                 815

Glu Gln Ile Gln Leu Lys Gly Thr Val Tyr Asn Tyr Arg Thr
820                 825                 830

Ser Gly Met Gln Phe Cys Val Lys Met Ser Ala Val Glu Gly
835                 840                 845

Ile Cys Thr Ser Glu Ser Pro Val Ile Asp His Gln Gly Thr
850                 855                 860

Lys Ser Ser Lys Cys Val Arg Gln Lys Val Glu Gly Ser Ser
865                 870

Ser His Leu Val Thr Phe Thr Val Leu Pro Leu Glu Ile Gly
875                 880                 885

Leu His Asn Ile Asn Phe Ser Leu Glu Thr Trp Phe Gly Lys
890                 895                 900

Glu Ile Leu Val Lys Thr Leu Arg Val Val Pro Glu Gly Val
905                 910                 915

Lys Arg Glu Ser Tyr Ser Gly Val Thr Leu Asp Pro Arg Gly
920                 925                 930

Ile Tyr Gly Thr Ile Ser Arg Arg Lys Glu Phe Pro Tyr Arg
935                 940
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Ile Pro Leu Asp Leu Val Pro Lys Thr Glu Ile Lys Arg Ile
945                 950                 955

Leu Ser Val Lys Gly Leu Leu Val Gly Glu Ile Leu Ser Ala
960                 965                 970

Val Leu Ser Gln Glu Gly Ile Asn Ile Leu Thr His Leu Pro
975                 980                 985

Lys Gly Ser Ala Glu Ala Glu Leu Met Ser Val Val Pro Val
990                 995                 1000

Phe Tyr Val Phe His Tyr Leu Glu Thr Gly Asn His Trp Asn
1005                1010

Ile Phe His Ser Asp Pro Leu Ile Glu Lys Gln Lys Leu Lys
1015                1020                1025

Lys Lys Leu Lys Glu Gly Met Leu Ser Ile Met Ser Tyr Arg
1030                1035                1040

Asn Ala Asp Tyr Ser Tyr Ser Val Trp Lys Gly Gly Ser Ala
1045                1050                1055

Ser Thr Trp Leu Thr Ala Phe Ala Leu Arg Val Leu Gly Gln
1060                1065                1070

Val Asn Lys Tyr Val Glu Gln Asn Gln Asn Ser Ile Cys Asn
1075                1080

Ser Leu Leu Trp Leu Val Glu Asn Tyr Gln Leu Asp Asn Gly
1085                1090                1095

Ser Phe Lys Glu Asn Ser Gln Tyr Gln Pro Ile Lys Leu Gln
1100                1105                1110

Gly Thr Leu Pro Val Glu Ala Arg Glu Asn Ser Leu Tyr Leu
1115                1120                1125

Thr Ala Phe Thr Val Ile Gly Ile Arg Lys Ala Phe Asp Ile
1130                1135                1140

Cys Pro Leu Val Lys Ile Asp Thr Ala Leu Ile Lys Ala Asp
1145                1150

Asn Phe Leu Leu Glu Asn Thr Leu Pro Ala Gln Ser Thr Phe
1155                1160                1165

Thr Leu Ala Ile Ser Ala Tyr Ala Leu Ser Leu Gly Asp Lys
1170                1175                1180

Thr His Pro Gln Phe Arg Ser Ile Val Ser Ala Leu Lys Arg
1185                1190                1195

Glu Ala Leu Val Lys Gly Asn Pro Pro Ile Tyr Arg Phe Trp
1200                1205                1210

Lys Asp Asn Leu Gln His Lys Asp Ser Ser Val Pro Asn Thr
1215                1220

Gly Thr Ala Arg Met Val Glu Thr Thr Ala Tyr Ala Leu Leu
1225                1230                1235

Thr Ser Leu Asn Leu Lys Asp Ile Asn Tyr Val Asn Pro Val
1240                1245                1250

Ile Lys Trp Leu Ser Glu Glu Gln Arg Tyr Gly Gly Gly Phe
1255                1260                1265

Tyr Ser Thr Gln Asp Thr Ile Asn Ala Ile Glu Gly Leu Thr
1270                1275                1280

Glu Tyr Ser Leu Leu Val Lys Gln Leu Arg Leu Ser Met Asp
1285                1290

Ile Asp Val Ser Tyr Lys His Lys Gly Ala Leu His Asn Tyr
1295                1300                1305
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Lys Met Thr Asp Lys Asn Phe Leu Gly Arg Pro Val Glu Val
1310                1315                1320

Leu Leu Asn Asp Asp Leu Ile Val Ser Thr Gly Phe Gly Ser
1325                1330                1335

Gly Leu Ala Thr Val His Val Thr Thr Val Val His Lys Thr
1340                1345                1350

Ser Thr Ser Glu Glu Val Cys Ser Phe Tyr Leu Lys Ile Asp
1355                1360

Thr Gln Asp Ile Glu Ala Ser His Tyr Arg Gly Tyr Gly Asn
1365                1370                1375

Ser Asp Tyr Lys Arg Ile Val Ala Cys Ala Ser Tyr Lys Pro
1380                1385                1390

Ser Arg Glu Glu Ser Ser Ser Gly Ser Ser His Ala Val Met
1395                1400                1405

Asp Ile Ser Leu Pro Thr Gly Ile Ser Ala Asn Glu Glu Asp
1410                1415                1420

Leu Lys Ala Leu Val Glu Gly Val Asp Gln Leu Phe Thr Asp
1425                1430

Tyr Gln Ile Lys Asp Gly His Val Ile Leu Gln Leu Asn Ser
1435                1440                1445

Ile Pro Ser Ser Asp Phe Leu Cys Val Arg Phe Arg Ile Phe
1450                1455                1460

Glu Leu Phe Glu Val Gly Phe Leu Ser Pro Ala Thr Phe Thr
1465                1470                1475

Val Tyr Glu Tyr His Arg Pro Asp Lys Gln Cys Thr Met Phe
1480                1485                1490

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1495                1500

Ala Ala Cys Lys Cys Val Glu Ala Asp Cys Gly Gln Met Gln
1505                1510                1515

Glu Glu Leu Asp Leu Thr Ile Ser Ala Glu Thr Arg Lys Gln
1520                1525                1530

Thr Ala Cys Lys Pro Glu Ile Ala Tyr Ala Tyr Lys Val Ser
1535                1540                1545

Ile Thr Ser Ile Thr Val Glu Asn Val Phe Val Lys Tyr Lys
1550                1555                1560

Ala Thr Leu Leu Asp Ile Tyr Lys Thr Gly Glu Ala Val Ala
1565                1570

Glu Lys Asp Ser Glu Ile Thr Phe Ile Lys Lys Val Thr Cys
1575                1580                1585

Thr Asn Ala Glu Leu Val Lys Gly Arg Gln Tyr Leu Ile Met
1590                1595                1600

Gly Lys Glu Ala Leu Gln Ile Lys Tyr Asn Phe Ser Phe Arg
1605                1610                1615

Tyr Ile Tyr Pro Leu Asp Ser Leu Thr Trp Ile Glu Tyr Trp
1620                1625                1630

Pro Arg Asp Thr Thr Cys Ser Ser Cys Gln Ala Phe Leu Ala
1635                1640

Asn Leu Asp Glu Phe Ala Glu Asp Ile Phe Leu Asn Gly Cys
1645                1650                1655
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4059 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: Circular

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Apex-1 Eukaryotic Expression Vector

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ACGCGTTGAC ATTGATTATT GACTAGTTAT TAATAGTAAT CAATTACGGG 50

GTCATTAGTT CATAGCCCAT ATATGGAGTT CCGCGTTACA TAACTTACGG 100

TAAATGGCCC CGCCTGGCTG ACCGCCCAAC GACCCCCGCC CATTGACGTC 150

AATAATGACG TATGTTCCCA TAGTAACGCC AATAGGGACT TTCCATTGAC 200

GTCAATGGGT GGACTATTTA CGGTAAACTG CCCACTTGGC AGTACATCAA 250

GTGTATCATA TGCCAAGTAC GCCCCCTATT GACGTCAATG ACGGTAAATG 300

GCCCGCCTGG CATTATGCCC AGTACATGAC CTTATGGGAC TTTCCTACTT 350

GGCAGTACAT CTACGTATTA GTCATCGCTA TTACCATGGT GATGCGGTTT 400

TGGCAGTACA TCAATGGGCG TGGATAGCGG TTTGACTCAC GGGGATTTCC 450

AAGTCTCCAC CCCATTGACG TCAATGGGAG TTTGTTTTGG CACCAAAATC 500

AACGGGACTT TCCAAAATGT CGTAACAACT CCGCCCCATT GACGCAAATG 550

GGCGGTAGGC GTGTACGGTG GGAGGTCTAT ATAAGCAGAG CTCGTTTAGT 600

GAACCGTCAG AATTCTGTTG GGCTCGCGGT TGATTACAAA CTCTTCGCGG 650

TCTTTCCAGT ACTCTTGGAT CGGAAACCCG TCGGCCTCCG AACGGTACTC 700

CGCCACCGAG GGACCTGAGC GAGTCCGCAT CGACCGGATC GGAAAACCTC 750

TCGACTGTTG GGGTGAGTAC TCCCTCTCAA AAGCGGGCAT GACTTCTGCG 800

CTAAGATTGT CAGTTTCCAA AAACGAGGAG GATTTGATAT TCACCTGGCC 850

CGCGGTGATG CCTTTGAGGG TGGCCGCGTC CATCTGGTCA GAAAAGACAA 900

TCTTTTTGTT GTCAAGCTTG AGGTGTGGCA GGCTTGAGAT CTGGCCATAC 950

ACTTGAGTGA CAATGACATC CACTTTGCCT TTCTCTCCAC AGGTGTCCAC 1000

TCCCAGGTCC AACTGCAGGT CGACCGGCTT GGTACCGAGC TCGGATCCAC 1050

TAGTAACGGC CGCCAGTGTG CTGGAATTCT GCAGATATCC ATCACACTGG 1100

CGGCCGCTCG AGCATGCATC TAGAACTTGT TTATTGCAGC TTATAATGGT 1150

TACAAATAAA GCAATAGCAT CACAAATTTC ACAAATAAAG CATTTTTTTC 1200

ACTGCATTCT AGTTGTGGTT TGTCCAAACT CATCAATGTA TCTTATCATG 1250

TCTGGATCGA TCCCGCCATG GTATCAACGC CATATTTCTA TTTACAGTAG 1300

GGACCTCTTC GTTGTGTAGG TACCGCTGTA TTCCTAGGGA AATAGTAGAG 1350

GCACCTTGAA CTGTCTGCAT CAGCCATATA GCCCCCGCTG TTCGACTTAC 1400

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TAGTTGCTAG GGCTGTCTCC GAACTCATTA CACCCTCCAA AGTCAGAGCT 1500

GTAATTTCGC CATCAAGGGC AGCGAGGGCT TCTCCAGATA AAATAGCTTC 1550

TGCCGAGAGT CCCGTAAGGG TAGACACTTC AGCTAATCCC TCGATGAGGT 1600

CTACTAGAAT AGTCAGTGCG GCTCCCATTT TGAAAATTCA CTTACTTGAT 1650

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CAGCTTCAGA AGATGGCGGA GGGCCTCCAA CACAGTAATT TTCCTCCCGA 1700

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CTCCAGAGGG CGTGTGGTTT TGCAAGAGGA AGCAAAAGCC TCTCCACCCA 1850

GGCCTAGAAT GTTTCCACCC AATCATTACT ATGACAACAG CTGTTTTTTT 1900

TAGTATTAAG CAGAGGCCGG GGACCCCTGG GCCCGCTTAC TCTGGAGAAA 1950

AAGAAGAGAG GCATTGTAGA GGCTTCCAGA GGCAACTTGT CAAAACAGGA 2000

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GCTGACTAAT TTTTTTTATT TATGCAGAGG CCGAGGCCGC CTCGGCCTCT 2200

GAGCTATTCC AGAAGTAGTG AGGAGGCTTT TTTGGAGGCC TAGGCTTTTG 2250

CAAAAAGGAG CTCCCAGCAA AAGGCCAGGA ACCGTAAAAA GGCCGCGTTG 2300

CTGGCGTTTT TCCATAGGCT CCGCCCCCCT GACGAGCATC ACAAAAATCG 2350

ACGCTCAAGT CAGAGGTGGC GAAACCCGAC AGGACTATAA AGATACCAGG 2400

CGTTTCCCCC TGGAAGCTCC CTCGTGCGCT CTCCTGTTCC GACCCTGCCG 2450

CTTACCGGAT ACCTGTCCGC CTTTCTCCCT TCGGGAAGCG TGGCGCTTTC 2500

TCAATGCTCA CGCTGTAGGT ATCTCAGTTC GGTGTAGGTC GTTCGCTCCA 2550

AGCTGGGCTG TGTGCACGAA CCCCCCGTTC AGCCCGACCG CTGCGCCTTA 2600

TCCGGTAACT ATCGTCTTGA GTCCAACCCG GTAAGACACG ACTTATCGCC 2650

ACTGGCAGCA GCCACTGGTA ACAGGATTAG CAGAGCGAGG TATGTAGGCG 2700

GTGCTACAGA GTTCTTGAAG TGGTGGCCTA ACTACGGCTA CACTAGAAGG 2750

ACAGTATTTG GTATCTGCGC TCTGCTGAAG CCAGTTACCT TCGGAAAAAG 2800

AGTTGGTAGC TCTTGATCCG GCAAACAAAC CACCGCTGGT AGCGGTGGTT 2850

TTTTTGTTTG CAAGCAGCAG ATTACGCGCA GAAAAAAAGG ATCTCAAGAA 2900

GATCCTTTGA TCTTTTCTAC GGGGTCTGAC GCTCAGTGGA ACGAAAACTC 2950

ACGTTAAGGG ATTTTGGTCA TGAGATTATC AAAAAGGATC TTCACCTAGA 3000

TCCTTTTAAA TTAAAAATGA AGTTTTAAAT CAATCTAAAG TATATATGAG 3050

TAAACTTGGT CTGACAGTTA CCAATGCTTA ATCAGTGAGG CACCTATCTC 3100

AGCGATCTGT CTATTTCGTT CATCCATAGT TGCCTGACTC CCCGTCGTGT 3150

AGATAACTAC GATACGGGAG GGCTTACCAT CTGGCCCCAG TGCTGCAATG 3200

ATACCGCGAG ACCCACGCTC ACCGGCTCCA GATTTATCAG CAATAAACCA 3250

GCCAGCCGGA AGGGCCGAGC GCAGAAGTGG TCCTGCAACT TTATCCGCCT 3300

CCATCCAGTC TATTAATTGT TGCCGGGAAG CTAGAGTAAG TAGTTCGCCA 3350

GTTAATAGTT TGCGCAACGT TGTTGCCATT GCTACAGGCA TCGTGGTGTC 3400

ACGCTCGTCG TTTGGTATGG CTTCATTCAG CTCCGGTTCC CAACGATCAA 3450

GGCGAGTTAC ATGATCCCCC ATGTTGTGCA AAAAAGCGGT TAGCTCCTTC 3500

GGTCCTCCGA TCGTTGTCAG AAGTAAGTTG GCCGCAGTGT TATCACTCAT 3550

GGTTATGGCA GCACTGCATA ATTCTCTTAC TGTCATGCCA TCCGTAAGAT 3600

GCTTTTCTGT GACTGGTGAG TACTCAACCA AGTCATTCTG AGAATAGTGT 3650

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GCCACATAGC AGAACTTTAA AAGTGCTCAT CATTGGAAAA CGTTCTTCGG 3750

GGCGAAAACT CTCAAGGATC TTACCGCTGT TGAGATCCAG TTCGATGTAA 3800

CCCACTCGTG CACCCAACTG ATCTTCAGCA TCTTTTACTT TCACCAGCGT 3850

TTCTGGGTGA GCAAAAACAG GAAGGCAAAA TGCCGCAAAA AAGGGAATAA 3900

GGGCGACACG GAAATGTTGA ATACTCATAC TCTTCCTTTT TCAATATTAT 3950

TGAAGCATTT ATCAGGGTTA TTGTCTCATG AGCGGATACA TATTTGAATG 4000

TATTTAGAAA AATAAACAAA TAGGGGTTCC GCGCACATTT CCCCGAAAAG 4050

TGCCACCTG 4059

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8540 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: Circular

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Apex-3P Eukaryotic Expression Vector

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTGACCAATA CAAAACAAAA GCGCCCCTCG TACCAGCGAA GAAGGGGCAG 50

AGATGCCGTA GTCAGGTTTA GTTCGTCCGG CGGCGGGGGA TCTGTATGGT 100

GCACTCTCAG TACAATCTGC TCTGATGCCG CATAGTTAAG CCAGTATCTG 150

CTCCCTGCTT GTGTGTTGGA GGTCGCTGAG TAGTGCGCGA GCAAAATTTA 200

AGCTACAACA AGGCAAGGCT TGACCGACAA TTGCATGAAG AATCTGCTTA 250

GGGTTAGGCG TTTTGCGCTG CTTCGCGATG TACGGGCCAG ATATACGCGT 300

TGACATTGAT TATTGACTAG TTATTAATAG TAATCAATTA CGGGGTCATT 350

AGTTCATAGC CCATATATGG AGTTCCGCGT TACATAACTT ACGGTAAATG 400

GCCCGCCTGG CTGACCGCCC AACGACCCCC GCCCATTGAC GTCAATAATG 450

ACGTATGTTC CCATAGTAAC GCCAATAGGG ACTTTCCATT GACGTCAATG 500

GGTGGACTAT TTACGGTAAA CTGCCCACTT GGCAGTACAT CAAGTGTATC 550

ATATGCCAAG TACGCCCCCT ATTGACGTCA ATGACGGTAA ATGGCCCGCC 600

TGGCATTATG CCCAGTACAT GACCTTATGG GACTTTCCTA CTTGGCAGTA 650

CATCTACGTA TTAGTCATCG CTATTACCAT GGTGATGCGG TTTTGGCAGT 700

ACATCAATGG GCGTGGATAG CGGTTTGACT CACGGGGATT TCCAAGTCTC 750

CACCCCATTG ACGTCAATGG GAGTTTGTTT TGGCACCAAA ATCAACGGGA 800

CTTTCCAAAA TGTCGTAACA ACTCCGCCCC ATTGACGCAA ATGGGCGGTA 850

GGCGTGTACG GTGGGAGGTC TATATAAGCA GAGCTCGTTT AGTGAACCGT 900

CAGAATTCTG TTGGGCTCGC GGTTGATTAC AAACTCTTCG CGGTCTTTCC 950

AGTACTCTTG GATCGGAAAC CCGTCGGCCT CCGAACGGTA CTCCGCCACC 1000

GAGGGACCTG AGCGAGTCCG CATCGACCGG ATCGGAAAAC CTCTCGACTG 1050

TTGGGGTGAG TACTCCCTCT CAAAAGCGGG CATGACTTCT GCGCTAAGAT 1100

TGTCAGTTTC CAAAAACGAG GAGGATTTGA TATTCACCTG GCCCGCGGTG 1150

ATGCCTTTGA GGGTGGCCGC GTCCATCTGG TCAGAAAAGA CAATCTTTTT 1200

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GTTGTCAAGC TTGAGGTGTG GCAGGCTTGA GATCTGGCCA TACACTTGAG 1250

TGACAATGAC ATCCACTTTG CCTTTCTCTC CACAGGTGTC CACTCCCAGG 1300

TCCAACTGCA GGTCGACCGG CTTGGTACCG AGCTCGGATC CTCTAGAGTC 1350

GACCTGCAGG CATGCAAGCT TGGCACTGGC CGTCGTTTTA CAACGTCGTG 1400

ACTGGGAAAA CCCTGGCGTT ACCCAACTTA ATCGCCTTGC AGCACATCCC 1450

CCTTTCGCCA GCTGGCGTAA TAGCGAAGAG GCCCGCACCG ATCCAGACAT 1500

GATAAGATAC ATTGATGAGT TTGGACAAAC CACAACTAGA ATGCAGTGAA 1550

AAAAATGCTT TATTTGTGAA ATTTGTGATG CTATTGCTTT ATTTGTAACC 1600

ATTATAAGCT GCAATAAACA AGTTAACAAC AACAATTGCA TTCATTTTAT 1650

GTTTCAGGTT CAGGGGGAGG TGTGGGAGGT TTTTTAAAGC AAGTAAAACC 1700

TCTACAAATG TGGTATGGCT GATTATGATC CCCAGGAAGC TCCTCTGTGT 1750

CCTCATAAAC CCTAACCTCC TCTACTTGAG AGGACATTCC AATCATAGGC 1800

TGCCCATCCA CCCTCTGTGT CCTCCTGTTA ATTAGGTCAC TTAACAAAAA 1850

GGAAATTGGG TAGGGGTTTT TCACAGACCG CTTTCTAAGG GTAATTTTAA 1900

AATATCTGGG AAGTCCCTTC CACTGCTGTG TTCCAGAAGT GTTGGTAAAC 1950

AGCCCACAAA TGTCAACAGC AGAAACATAC AAGCTGTCAG CTTTGCACAA 2000

GGGCCCAACA CCCTGCTCAT CAAGAAGCAC TGTGGTTGCT GTGTTAGTAA 2050

TGTGCAAAAC AGGAGGCACA TTTTCCCCAC CTGTGTAGGT TCCAAAATAT 2100

CTAGTGTTTT CATTTTTACT TGGATCAGGA ACCCAGCACT CCACTGGATA 2150

AGCATTATCC TTATCCAAAA CAGCCTTGTG GTCAGTGTTC ATCTGCTGAC 2200

TGTCAACTGT AGCATTTTTT GGGGTTACAG TTTGAGCAGG ATATTTGGTC 2250

CTGTAGTTTG CTAACACACC CTGCAGCTCC AAAGGTTCCC CACCAACAGC 2300

AAAAAAATGA AAATTTGACC CTTGAATGGG TTTTCCAGCA CCATTTTCAT 2350

GAGTTTTTTG TGTCCCTGAA TGCAAGTTTA ACATAGCAGT TACCCCAATA 2400

ACCTCAGTTT TAACAGTAAC AGCTTCCCAC ATCAAAATAT TTCCACAGGT 2450

TAAGTCCTCA TTTGTAGAAT TCGCCAGCAC AGTGGTCGAC CCTGTGGATG 2500

TGTGTCACTT AGGGTGTGGA AAGTCCCCAG GCTCCCCAGC AGGCAGAAGT 2550

ATGCAAAGCA TGCATCTCAA TTAGTCAGCA ACCAGGTGTG GAAAGTCCCC 2600

AGGCTCCCCA GCAGGCAGAA GTATGCAAAG CATGCATCTC AATTAGTCAG 2650

CAACCATAGT CCCGCCCCTA ACTCCGCCCA TCCCGCCCCT AACTCCGCCC 2700

AGTTCCGCCC ATTCTCCGCC CCATGGCTGA CTAATTTTTT TTATTTATGC 2750

AGAGGCCGAG GCCGCCTCGG CCTCTGAGCT ATTCCAGAAG TAGTGAGGAG 2800

GCTTTTTTGG AGGCCTAGGC TTTTGCAAAA GCTTACCATG ACCGAGTACA 2850

AGCCCACGGT GCGCCTCGCC ACCCGCGACG ACGTCCCCCG GGCCGTACGC 2900

ACCCTCGCCG CCGCGTTCGC CGACTACCCC GCCACGCGCC ACACCGTCGA 2950

CCCGGACCGC CACATCGAGC GGGTCACCGA GCTGCAAGAA CTCTTCCTCA 3000

CGCGCGTCGG GCTCGACATC GGCAAGGTGT GGGTCGCGGA CGACGGCGCC 3050

GCGGTGGCGG TCTGGACCAC GCCGGAGAGC GTCGAAGCGG GGGCGGTGTT 3100

CGCCGAGATC GGCCCGCGCA TGGCCGAGTT GAGCGGTTCC CGGCTGGCCG 3150

CGCAGCAACA GATGGAAGGC CTCCTGGCGC CGCACCGGCC CAAGGAGCCC 3200

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GCGTGGTTCC TGGCCACCGT CGGCGTCTCG CCCGACCACC AGGGCAAGGG 3250

TCTGGGCAGC GCCGTCGTGC TCCCCGGAGT GGAGGCGGCC GAGCGCGCCG 3300

GGGTGCCCGC CTTCCTGGAG ACCTCCGCGC CCCGCAACCT CCCCTTCTAC 3350

GAGCGGCTCG GCTTCACCGT CACCGCCGAC GTCGAGTGCC CGAAGGACCG 3400

CGCGACCTGG TGCATGACCC GCAAGCCCGG TGCCTGACGC CCGCCCCACG 3450

ACCCGCAGCG CCCGACCGAA AGGAGCGCAC GACCCCATGC ATCGATAAAA 3500

TAAAAGATTT TATTTAGTCT CCAGAAAAAG GGGGGAATGA AAGACCCCAC 3550

CTGTAGGTTT GGCAAGCTAG AACTTGTTTA TTGCAGCTTA TAATGGTTAC 3600

AAATAAAGCA ATAGCATCAC AAATTTCACA AATAAAGCAT TTTTTTCACT 3650

GCATTCTAGT TGTGGTTTGT CCAAACTCAT CAATGTATCT TATCATGTCT 3700

GGATCGATCC CGCCATGGTA TCAACGCCAT ATTTCTATTT ACAGTAGGGA 3750

CCTCTTCGTT GTGTAGGTAC CCCGGGTTCG AAATCGAATT CGCCAATGAC 3800

AAGACGCTGG GCGGGGTTTG TGTCATCATA GAACTAAAGA CATGCAAATA 3850

TATTTCTTCC GGGGACACCG CCAGCAAACG CGAGCAACGG GCCACGGGGA 3900

TGAAGCAGCC CGGCGGCACC TCGCTAACGG ATTCACCACT CCAAGAATTG 3950

GAGCCAATCA ATTCTTGCGG AGAACTGTGA ATGCGCAAAC CAACCCTTGG 4000

CAGAACATAT CCATCGCGTC CGCCATCTCC AGCAGCCGCA CGCGGCGCAT 4050

CTCGGGGCCG ACGCGCTGGG CTACGTCTTG CTGGCGTTCG CGACGCGAGG 4100

CTGGATGGCC TTCCCCATTA TGATTCTTCT CGCTTCCGGC GGCATCGGGA 4150

TGCCCGCGTT GCAGGCCATG CTGTCCAGGC AGGTAGATGA CGACCATCAG 4200

GGACAGCTTC AAGGATCGCT CGCGGCTCTT ACCAGCGCCA GCAAAAGGCC 4250

AGGAACCGTA AAAAGGCCGC GTTGCTGGCG TTTTTCCATA GGCTCCGCCC 4300

CCCTGACGAG CATCACAAAA ATCGACGCTC AAGTCAGAGG TGGCGAAACC 4350

CGACAGGACT ATAAAGATAC CAGGCGTTTC CCCCTGGAAG CTCCCTCGTG 4400

CGCTCTCCTG TTCCGACCCT GCCGCTTACC GGATACCTGT CCGCCTTTCT 4450

CCCTTCGGGA AGCGTGGCGC TTTCTCATAG CTCACGCTGT AGGTATCTCA 4500

GTTCGGTGTA GGTCGTTCGC TCCAAGCTGG GCTGTGTGCA CGAACCCCCC 4550

GTTCAGCCCG ACCGCTGCGC CTTATCCGGT AACTATCGTC TTGAGTCCAA 4600

CCCGGTAAGA CACGACTTAT CGCCACTGGC AGCAGCCACT GGTAACAGGA 4650

TTAGCAGAGC GAGGTATGTA GGCGGTGCTA CAGAGTTCTT GAAGTGGTGG 4700

CCTAACTACG GCTACACTAG AAGGACAGTA TTTGGTATCT GCGCTCTGCT 4750

GAAGCCAGTT ACCTTCGGAA AAAGAGTTGG TAGCTCTTGA TCCGGCAAAC 4800

AAACCACCGC TGGTAGCGGT GGTTTTTTTG TTTGCAAGCA GCAGATTACG 4850

CGCAGAAAAA AAGGATCTCA AGAAGATCCT TTGATCTTTT CTACGGGGTC 4900

TGACGCTCAG TGGAACGAAA ACTCACGTTA AGGGATTTTG GTCATGAGAT 4950

TATCAAAAAG GATCTTCACC TAGATCCTTT TAAATTAAAA ATGAAGTTTT 5000

AAATCAATCT AAAGTATATA TGAGTAAACT TGGTCTGACA GTTACCAATG 5050

CTTAATCAGT GAGGCACCTA TCTCAGCGAT CTGTCTATTT CGTTCATCCA 5100

TAGTTGCCTG ACTCCCCGTC GTGTAGATAA CTACGATACG GGAGGGCTTA 5150

CCATCTGGCC CCAGTGCTGC AATGATACCG CGAGACCCAC GCTCACCGGC 5200

-continued

TCCAGATTTA TCAGCAATAA ACCAGCCAGC CGGAAGGGCC GAGCGCAGAA 5250

GTGGTCCTGC AACTTTATCC GCCTCCATCC AGTCTATTAA TTGTTGCCGG 5300

GAAGCTAGAG TAAGTAGTTC GCCAGTTAAT AGTTTGCGCA ACGTTGTTGC 5350

CATTGCTGCA GGCATCGTGG TGTCACGCTC GTCGTTTGGT ATGGCTTCAT 5400

TCAGCTCCGG TTCCCAACGA TCAAGGCGAG TTACATGATC CCCCATGTTG 5450

TGCAAAAAAG CGGTTAGCTC CTTCGGTCCT CCGATCGTTG TCAGAAGTAA 5500

GTTGGCCGCA GTGTTATCAC TCATGGTTAT GGCAGCACTG CATAATTCTC 5550

TTACTGTCAT GCCATCCGTA AGATGCTTTT CTGTGACTGG TGAGTACTCA 5600

ACCAAGTCAT TCTGAGAATA GTGTATGCGG CGACCGAGTT GCTCTTGCCC 5650

GGCGTCAACA CGGGATAATA CCGCGCCACA TAGCAGAACT TTAAAAGTGC 5700

TCATCATTGG AAAACGTTCT TCGGGGCGAA AACTCTCAAG GATCTTACCG 5750

CTGTTGAGAT CCAGTTCGAT GTAACCCACT CGTGCACCCA ACTGATCTTC 5800

AGCATCTTTT ACTTTCACCA GCGTTTCTGG GTGAGCAAAA ACAGGAAGGC 5850

AAAATGCCGC AAAAAAGGGA ATAAGGGCGA CACGGAAATG TTGAATACTC 5900

ATACTCTTCC TTTTTCAATA TTATTGAAGC ATTTATCAGG GTTATTGTCT 5950

CATGAGCGGA TACATATTTG AATGTATTTA GAAAAATAAA CAAATAGGGG 6000

TTCCGCGCAC ATTTCCCCGA AAAGTGCCAC CTGACGTCTA AGAAACCATT 6050

ATTATCATGA CATTAACCTA TAAAAATAGG CGTATCACGA GGCCCTTTCG 6100

TCTTCAAGAA TTCTCATGTT TGACAGCTTA TCGTAGACAT CATGCGTGCT 6150

GTTGGTGTAT TTCTGGCCAT CTGTCTTGTC ACCATTTTCG TCCTCCCAAC 6200

ATGGGGCAAT TGGGCATACC CATGTTGTCA CGTCACTCAG CTCCGCGCTC 6250

AACACCTTCT CGCGTTGGAA AACATTAGCG ACATTTACCT GGTGAGCAAT 6300

CAGACATGCG ACGGCTTTAG CCTGGCCTCC TTAAATTCAC CTAAGAATGG 6350

GAGCAACCAG CAGGAAAAGG ACAAGCAGCG AAAATTCACG CCCCCTTGGG 6400

AGGTGGCGGC ATATGCAAAG GATAGCACTC CCACTCTACT ACTGGGTATC 6450

ATATGCTGAC TGTATATGCA TGAGGATAGC ATATGCTACC CGGATACAGA 6500

TTAGGATAGC ATATACTACC CAGATATAGA TTAGGATAGC ATATGCTACC 6550

CAGATATAGA TTAGGATAGC CTATGCTACC CAGATATAAA TTAGGATAGC 6600

ATATACTACC CAGATATAGA TTAGGATAGC ATATGCTACC CAGATATAGA 6650

TTAGGATAGC CTATGCTACC CAGATATAGA TTAGGATAGC ATATGCTACC 6700

CAGATATAGA TTAGGATAGC ATATGCTATC CAGATATTTG GGTAGTATAT 6750

GCTACCCAGA TATAAATTAG GATAGCATAT ACTACCCTAA TCTCTATTAG 6800

GATAGCATAT GCTACCCGGA TACAGATTAG GATAGCATAT ACTACCCAGA 6850

TATAGATTAG GATAGCATAT GCTACCCAGA TATAGATTAG GATAGCCTAT 6900

GCTACCCAGA TATAAATTAG GATAGCATAT ACTACCCAGA TATAGATTAG 6950

GATAGCATAT GCTACCCAGA TATAGATTAG GATAGCCTAT GCTACCCAGA 7000

TATAGATTAG GATAGCATAT GCTATCCAGA TATTTGGGTA GTATATGCTA 7050

CCCATGGCAA CATTAGCCCA CCGTGCTCTC AGCGACCTCG TGAATATGAG 7100

GACCAACAAC CCTGTGCTTG GCGCTCAGGC GCAAGTGTGT GTAATTTGTC 7150

CTCCAGATCG CAGCAATCGC GCCCCTATCT TGGCCCGCCC ACCTACTTAT 7200

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GCAGGTATTC CCCGGGGTGC CATTAGTGGT TTTGTGGGCA AGTGGTTTGA              7250

CCGCAGTGGT TAGCGGGGTT ACAATCAGCC AAGTTATTAC ACCCTTATTT              7300

TACAGTCCAA AACCGCAGGG CGGCGTGTGG GGGCTGACGC GTGCCCCCAC              7350

TCCACAATTT CAAAAAAAAG AGTGGCCACT TGTCTTTGTT TATGGGCCCC              7400

ATTGGCGTGG AGCCCCGTTT AATTTTCGGG GGTGTTAGAG ACAACCAGTG              7450

GAGTCCGCTG CTGTCGGCGT CCACTCTCTT TCCCCTTGTT ACAAATAGAG              7500

TGTAACAACA TGGTTCACCT GTCTTGGTCC CTGCCTGGGA CACATCTTAA              7550

TAACCCCAGT ATCATATTGC ACTAGGATTA TGTGTTGCCC ATAGCCATAA              7600

ATTCGTGTGA GATGGACATC CAGTCTTTAC GGCTTGTCCC CACCCCATGG              7650

ATTTCTATTG TTAAAGATAT TCAGAATGTT TCATTCCTAC ACTAGTATTT              7700

ATTGCCCAAG GGGTTTGTGA GGGTTATATT GGTGTCATAG CACAATGCCA              7750

CCACTGAACC CCCCGTCCAA ATTTTATTCT GGGGGCGTCA CCTGAAACCT              7800

TGTTTTCGAG CACCTCACAT ACACCTTACT GTTCACAACT CAGCAGTTAT              7850

TCTATTAGCT AAACGAAGGA GAATGAAGAA GCAGGCGAAG ATTCAGGAGA              7900

GTTCACTGCC CGCTCCTTGA TCTTCAGCCA CTGCCCTTGT GACTAAAATG              7950

GTTCACTACC CTCGTGGAAT CCTGACCCCA TGTAAATAAA ACCGTGACAG              8000

CTCATGGGGT GGGAGATATC GCTGTTCCTT AGGACCCTTT TACTAACCCT              8050

AATTCGATAG CATATGCTTC CCGTTGGGTA ACATATGCTA TTGAATTAGG              8100

GTTAGTCTGG ATAGTATATA CTACTACCCG GGAAGCATAT GCTACCCGTT              8150

TAGGGTTAAC AAGGGGGCCT TATAAACACT ATTGCTAATG CCCTCTTGAG              8200

GGTCCGCTTA TCGGTAGCTA CACAGGCCCC TCTGATTGAC GTTGGTGTAG              8250

CCTCCCGTAG TCTTCCTGGG CCCCTGGGAG GTACATGTCC CCCAGCATTG              8300

GTGTAAGAGC TTCAGCCAAG AGTTACACAT AAAGGCAATG TTGTGTTGCA              8350

GTCCACAGAC TGCAAAGTCT GCTCCAGGAT GAAAGCCACT CAGTGTTGGC              8400

AAATGTGCAC ATCCATTTAT AAGGATGTCA ACTACAGTCA GAGAACCCCT              8450

TTGTGTTTGG TCCCCCCCCG TGTCACATGT GGAACAGGGC CCAGTTGGCA              8500

AGTTGTACCA ACCAACTGAA GGGATTACAT GCACTGCCCC                         8540
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 bases
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer UDEC690

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
CGCCTGCAGG ACATCCAGAT GACTCAGTCT                                    30
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 bases
(B) TYPE: Nucleic Acid

-continued

(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer UDEC395

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCCAAGCTTA CTGGATGGTG GGAAGATGGA 30

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1M1 scFv (murine)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
ATG GCC GAC ATC CAG ATG ACT CAG TCT CCA                          30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10

GCT TCA CTG TCT GCA TCT GTG GGA GAA ACT                          60
Ala Ser Leu Ser Ala Ser Val Gly Glu Thr
15                  20

GTC ACC ATC ACA TGT GGA GCA AGT GAG AAT                          90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
25                  30

ATT TAC GGT GCT TTA AAT TGG TAT CAG CGG                          120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Arg
35                  40

AAA CAG GGA AAA TCT CCT CAG CTC CTG ATC                          150
Lys Gln Gly Lys Ser Pro Gln Leu Leu Ile
45                  50

TAT GGT GCA ACC AAC TTG GCA GAT GGC ATG                          180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Met
55                  60

TCA TCG AGG TTC AGT GGC AGT GGA TCT GGT                          210
Ser Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

AGA CAG TAT TAT CTC AAG ATC AGT AGC CTG                          240
Arg Gln Tyr Tyr Leu Lys Ile Ser Ser Leu
75                  80

CAT CCT GAC GAT GTT GCA ACG TAT TAC TGT                          270
His Pro Asp Asp Val Ala Thr Tyr Tyr Cys
85                  90

CAA AAT GTG TTA AAT ACT CCT CTC ACG TTC                          300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                 100

GGT GCT GGG ACC AAG TTG GAG CTG AAA CGG                          330
Gly Ala Gly Thr Lys Leu Glu Leu Lys Arg
105                 110

ACC GGA GGT GGC GGG TCG GGT GGC GGG GGA                          360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120

TCG GGT GGC GGA GGG TCG CAG GTT CAG CTG                          390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130
```

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CAG CAG TCT GGA GCC GAG CTG ATG AAG CCT                                  420
Gln Gln Ser Gly Ala Glu Leu Met Lys Pro
135                 140

GGG GCC TCA GTG AAG ATG TCC TGC AAG GCT                                  450
Gly Ala Ser Val Lys Met Ser Cys Lys Ala
145                 150

ACT GGC TAC ATA TTC AGT AAC TAC TGG ATA                                  480
Thr Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAG TGG ATA AAG CAG AGG CCT GGA CAT GGC                                  510
Gln Trp Ile Lys Gln Arg Pro Gly His Gly
165                 170

CTT GAG TGG ATT GGT GAG ATT TTA CCT GGA                                  540
Leu Glu Trp Ile Gly Glu Ile Leu Pro Gly
175                 180

AGT GGT TCT ACT GAG TAC ACT GAG AAC TTC                                  570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
185                 190

AAG GAC AAG GCC GCA TTC ACT GCA GAT ACA                                  600
Lys Asp Lys Ala Ala Phe Thr Ala Asp Thr
195                 200

TCC TCC AAC ACA GCC TAC ATG CAA CTC AGC                                  630
Ser Ser Asn Thr Ala Tyr Met Gln Leu Ser
205                 210

AGC CTG ACA TCA GAG GAC TCT GCC GTC TAT                                  660
Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr
215                 220

TAC TGT GCA AGA TAT TTC TTC GGT AGT AGC                                  690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230

CCC AAC TGG TAC TTC GAT GTC TGG GGC GCA                                  720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Ala
235                 240

GGG ACC ACG GTC ACC GTC TCC TCA TGA                                      747
Gly Thr Thr Val Thr Val Ser Ser
245
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 scFv CB (humanized)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                                   30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                                   60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                                   90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CGT                                  120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Arg
35                  40
```

-continued

```
AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                                  150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                                  180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                                  210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                                  240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                                  270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                                  300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                                  330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                                  360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                                  390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                                  420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                                  450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                                  480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                                  510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                                  540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                                  570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                                  600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                                  630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                                  660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                                  690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                                  720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
235                 240
```

-continued

```
GGA ACC CTG GTC ACT GTC TCG AGC TGA                                    747
Gly Thr Leu Val Thr Val Ser Ser
245

(2) INFORMATION FOR SEQ ID NO:9:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH:  726 base pairs
          (B) TYPE:  Nucleic Acid
          (C) STRANDEDNESS:  Double
          (D) TOPOLOGY:  linear

    (ii) MOLECULE TYPE:  Other nucleic acid
          (A) DESCRIPTION:5G1.1M1 VL HuK (chimeric light chain)

    (xi) SEQUENCE DESCRIPTION:  SEQ ID NO:9:

ATG GGA ATC CAA GGA GGG TCT GTC CTG TTC                                 30
Met Gly Ile Gln Gly Gly Ser Val Leu Phe
-25                 -20

GGG CTG CTG CTC GTC CTG GCT GTC TTC TGC                                 60
Gly Leu Leu Leu Val Leu Ala Val Phe Cys
-15                 -10

CAT TCA GGT CAT AGC CTG CAG GAC ATC CAG                                 90
His Ser Gly His Ser Leu Gln Asp Ile Gln
-5                   1                   5

ATG ACT CAG TCT CCA GCT TCA CTG TCT GCA                                 120
Met Thr Gln Ser Pro Ala Ser Leu Ser Ala
10                  15

TCT GTG GGA GAA ACT GTC ACC ATC ACA TGT                                 150
Ser Val Gly Glu Thr Val Thr Ile Thr Cys
20                  25

GGA GCA AGT GAG AAT ATT TAC GGT GCT TTA                                 180
Gly Ala Ser Glu Asn Ile Tyr Gly Ala Leu
30                  35

AAT TGG TAT CAG CGG AAA CAG GGA AAA TCT                                 210
Asn Trp Tyr Gln Arg Lys Gln Gly Lys Ser
40                  45

CCT CAG CTC CTG ATC TAT GGT GCA ACC AAC                                 240
Pro Gln Leu Leu Ile Tyr Gly Ala Thr Asn
50                  55

TTG GCA GAT GGC ATG TCA TCG AGG TTC AGT                                 270
Leu Ala Asp Gly Met Ser Ser Arg Phe Ser
60                  65

GGC AGT GGA TCT GGT AGA CAG TAT TAT CTC                                 300
Gly Ser Gly Ser Gly Arg Gln Tyr Tyr Leu
70                  75

AAG ATC AGT AGC CTG CAT CCT GAC GAT GTT                                 330
Lys Ile Ser Ser Leu His Pro Asp Asp Val
80                  85

GCA ACG TAT TAC TGT CAA AAT GTG TTA AAT                                 360
Ala Thr Tyr Tyr Cys Gln Asn Val Leu Asn
90                  95

ACT CCT CTC ACG TTC GGT GCT GGG ACC AAG                                 390
Thr Pro Leu Thr Phe Gly Ala Gly Thr Lys
100                 105

TTG GAG CTG AAA CGA ACT GTG GCT GCA CCA                                 420
Leu Glu Leu Lys Arg Thr Val Ala Ala Pro
110                 115

TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG                                 450
Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
120                 125
```

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```
CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG                                  480
Gln Leu Lys Ser Gly Thr Ala Ser Val Val
130                 135

TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG                                  510
Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu
140                 145

GCC AAA GTA CAG TGG AAG GTG GAT AAC GCC                                  540
Ala Lys Val Gln Trp Lys Val Asp Asn Ala
150                 155

CTC CAA TCG GGT AAC TCC CAG GAG AGT GTC                                  570
Leu Gln Ser Gly Asn Ser Gln Glu Ser Val
160                 165

ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC                                  600
Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
170                 175

AGC CTC AGC AGC ACC CTG ACG CTG AGC AAA                                  630
Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys
180                 185

GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC                                  660
Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
190                 195

TGC GAA GTC ACC CAT CAG GGC CTG AGC TCG                                  690
Cys Glu Val Thr His Gln Gly Leu Ser Ser
200                 205

CCC GTC ACA AAG AGC TTC AAC AGG GGA GAG                                  720
Pro Val Thr Lys Ser Phe Asn Arg Gly Glu
210                 215

TGT TAG                                                                  726
Cys
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 750 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1M1 VH +HuG1 (chimeric Fd)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
ATG AAA TGG AGC TGG GTT ATT CTC TTC CTC                                   30
Met Lys Trp Ser Trp Val Ile Leu Phe Leu
-15                 -10

CTG TCA GTA ACT GCA GGT GTC CAC TCC CAG                                   60
Leu Ser Val Thr Ala Gly Val His Ser Gln
-5                  1

GTT CAG CTG CAG CAG TCT GGA GCT GAG CTG                                   90
Val Gln Leu Gln Gln Ser Gly Ala Glu Leu
5                   10

ATG AAG CCT GGG GCC TCA GTG AAG ATG TCC                                  120
Met Lys Pro Gly Ala Ser Val Lys Met Ser
15                  20

TGC AAG GCT ACT GGC TAC ATA TTC AGT AAC                                  150
Cys Lys Ala Thr Gly Tyr Ile Phe Ser Asn
25                  30

TAC TGG ATA CAG TGG ATA AAG CAG AGG CCT                                  180
Tyr Trp Ile Gln Trp Ile Lys Gln Arg Pro
35                  40

GGA CAT GGC CTT GAG TGG ATT GGT GAG ATT                                  210
Gly His Gly Leu Glu Trp Ile Gly Glu Ile
45                  50
```

-continued

```
TTA CCT GGA AGT GGT TCT ACT GAG TAC ACT                          240
Leu Pro Gly Ser Gly Ser Thr Glu Tyr Thr
55                  60

GAG AAC TTC AAG GAC AAG GCC GCA TTC ACT                          270
Glu Asn Phe Lys Asp Lys Ala Ala Phe Thr
65                  70

GCA GAT ACA TCC TCC AAC ACA GCC TAC ATG                          300
Ala Asp Thr Ser Ser Asn Thr Ala Tyr Met
75                  80

CAA CTC AGC AGC CTG ACA TCA GAG GAC TCT                          330
Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser
85                  90

GCC GTC TAT TAC TGT GCA AGA TAT TTC TTC                          360
Ala Val Tyr Tyr Cys Ala Arg Tyr Phe Phe
95                 100

GGT AGT AGC CCC AAC TGG TAC TTC GAT GTC                          390
Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
105                 110

TGG GGC GCA GGG ACC ACG GTC ACC GTC TCC                          420
Trp Gly Ala Gly Thr Thr Val Thr Val Ser
115                 120

TCA GCC TCC ACC AAG GGC CCA TCG GTC TTC                          450
Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
125                 130

CCC CTG GCG CCC TCC TCC AAG AGC ACC TCT                          480
Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
135                 140

GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC                          510
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
145                 150

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG                          540
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
155                 160

TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC                          570
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
165                 170

GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC                          600
Val His Thr Phe Pro Ala Val Leu Gln Ser
175                 180

TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG                          630
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
185                 190

ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG                          660
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
195                 200

ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC                          690
Thr Tyr Ile Cys Asn Val Asn His Lys Pro
205                 210

AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG                          720
Ser Asn Thr Lys Val Asp Lys Lys Val Glu
215                 220

CCC AAA TCT TGT GAC AAA ACT CAC ACA TAA                          750
Pro Lys Ser Cys Asp Lys Thr His Thr
225
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 750 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

-continued

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 VH + IGHRL (Humanized Fd)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
ATG AAG TGG AGC TGG GTT ATT CTC TTC CTC                          30
Met Lys Trp Ser Trp Val Ile Leu Phe Leu
-15                 -10

CTG TCA GTA ACT GCC GGC GTC CAC TCC CAA                          60
Leu Ser Val Thr Ala Gly Val His Ser Gln
-5                  1

GTC CAA CTG GTG CAA TCC GGC GCC GAG GTC                          90
Val Gln Leu Val Gln Ser Gly Ala Glu Val
5                   10

AAG AAG CCA GGG GCC TCA GTC AAA GTG TCC                         120
Lys Lys Pro Gly Ala Ser Val Lys Val Ser
15                  20

TGT AAA GCT AGC GGC TAT ATT TTT TCT AAT                         150
Cys Lys Ala Ser Gly Tyr Ile Phe Ser Asn
25                  30

TAT TGG ATT CAA TGG GTG CGT CAG GCC CCC                         180
Tyr Trp Ile Gln Trp Val Arg Gln Ala Pro
35                  40

GGG CAG GGC CTG GAA TGG ATG GGT GAG ATC                         210
Gly Gln Gly Leu Glu Trp Met Gly Glu Ile
45                  50

TTA CCG GGC TCT GGT AGC ACC GAA TAT GCC                         240
Leu Pro Gly Ser Gly Ser Thr Glu Tyr Ala
55                  60

CAA AAA TTC CAG GGC CGT GTT ACT ATG ACT                         270
Gln Lys Phe Gln Gly Arg Val Thr Met Thr
65                  70

GCG GAC ACT TCG ACT AGT ACA GCC TAC ATG                         300
Ala Asp Thr Ser Thr Ser Thr Ala Tyr Met
75                  80

GAG CTC TCC AGC CTG CGA TCG GAG GAC ACG                         330
Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr
85                  90

GCC GTC TAT TAT TGC GCG CGT TAT TTT TTT                         360
Ala Val Tyr Tyr Cys Ala Arg Tyr Phe Phe
95                 100

GGT TCT AGC CCG AAT TGG TAT TTT GAT GTT                         390
Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
105                 110

TGG GGT CAA GGA ACC CTG GTC ACT GTC TCG                         420
Trp Gly Gln Gly Thr Leu Val Thr Val Ser
115                 120

AGC GCC TCC ACC AAG GGC CCA TCG GTC TTC                         450
Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
125                 130

CCC CTG GCG CCC TCC TCC AAG AGC ACC TCT                         480
Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
135                 140

GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC                         510
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
145                 150

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG                         540
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
155                 160
```

-continued

```
TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC                                  570
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
165                 170

GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC                                  600
Val His Thr Phe Pro Ala Val Leu Gln Ser
175                 180

TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG                                  630
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
185                 190

ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG                                  660
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
195                 200

ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC                                  690
Thr Tyr Ile Cys Asn Val Asn His Lys Pro
205                 210

AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG                                  720
Ser Asn Thr Lys Val Asp Lys Lys Val Glu
215                 220

CCC AAA TCT TGT GAC AAA ACT CAC ACA TAA                                  750
Pro Lys Ser Cys Asp Lys Thr His Thr
225                 230
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 750 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 VH + IGHRLC (Humanized Fd)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
ATG AAG TGG AGC TGG GTT ATT CTC TTC CTC                                   30
Met Lys Trp Ser Trp Val Ile Leu Phe Leu
-15                 -10

CTG TCA GTA ACT GCC GGC GTC CAC TCC CAA                                   60
Leu Ser Val Thr Ala Gly Val His Ser Gln
-5                   1

GTC CAA CTG GTG CAA TCC GGC GCC GAG GTC                                   90
Val Gln Leu Val Gln Ser Gly Ala Glu Val
5                   10

AAG AAG CCA GGG GCC TCA GTC AAA GTG TCC                                  120
Lys Lys Pro Gly Ala Ser Val Lys Val Ser
15                  20

TGT AAA GCT AGC GGC TAT ATT TTT TCT AAT                                  150
Cys Lys Ala Ser Gly Tyr Ile Phe Ser Asn
25                  30

TAT TGG ATT CAA TGG GTG CGT CAG GCC CCC                                  180
Tyr Trp Ile Gln Trp Val Arg Gln Ala Pro
35                  40

GGG CAG GGC CTG GAA TGG ATG GGT GAG ATC                                  210
Gly Gln Gly Leu Glu Trp Met Gly Glu Ile
45                  50

TTA CCG GGC TCT GGT AGC ACC GAA TAT ACC                                  240
Leu Pro Gly Ser Gly Ser Thr Glu Tyr Thr
55                  60

GAA AAT TTT AAA GAC CGT GTT ACT ATG ACG                                  270
Glu Asn Phe Lys Asp Arg Val Thr Met Thr
65                  70
```

-continued

```
CGT GAC ACT TCG ACT AGT ACA GTA TAC ATG                                  300
Arg Asp Thr Ser Thr Ser Thr Val Tyr Met
75                  80

GAG CTC TCC AGC CTG CGA TCG GAG GAC ACG                                 330
Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr
85                  90

GCC GTC TAT TAT TGC GCG CGT TAT TTT TTT                                 360
Ala Val Tyr Tyr Cys Ala Arg Tyr Phe Phe
95                 100

GGT TCT AGC CCG AAT TGG TAT TTT GAT GTT                                 390
Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
105                 110

TGG GGT CAA GGA ACC CTG GTC ACT GTC TCG                                 420
Trp Gly Gln Gly Thr Leu Val Thr Val Ser
115                 120

AGC GCC TCC ACC AAG GGC CCA TCG GTC TTC                                 450
Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
125                 130

CCC CTG GCG CCC TCC TCC AAG AGC ACC TCT                                 480
Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
135                 140

GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC                                 510
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
145                 150

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG                                 540
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
155                 160

TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC                                 570
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
165                 170

GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC                                 600
Val His Thr Phe Pro Ala Val Leu Gln Ser
175                 180

TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG                                 630
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
185                 190

ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG                                 660
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
195                 200

ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC                                 690
Thr Tyr Ile Cys Asn Val Asn His Lys Pro
205                 210

AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG                                 720
Ser Asn Thr Lys Val Asp Lys Lys Val Glu
215                 220

CCC AAA TCT TGT GAC AAA ACT CAC ACA TAA                                 750
Pro Lys Ser Cys Asp Lys Thr His Thr
225                 230
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 726 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 VL +KLV56
(Humanized light chain)

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
ATG GGA ATC CAA GGA GGG TCT GTC CTG TTC                          30
Met Gly Ile Gln Gly Gly Ser Val Leu Phe
-25                 -20

GGG CTG CTG CTC GTC CTG GCT GTC TTC TGC                          60
Gly Leu Leu Leu Val Leu Ala Val Phe Cys
-15                 -10

CAT TCA GGT CAT AGC CTG CAG GAT ATC CAG                          90
His Ser Gly His Ser Leu Gln Asp Ile Gln
-5                   1               5

ATG ACC CAG TCC CCG TCC TCC CTG TCC GCC                         120
Met Thr Gln Ser Pro Ser Ser Leu Ser Ala
10                  15

TCT GTG GGC GAT AGG GTC ACC ATC ACC TGC                         150
Ser Val Gly Asp Arg Val Thr Ile Thr Cys
20                  25

GGC GCC AGC GAA AAC ATC TAT GGC GCG CTG                         180
Gly Ala Ser Glu Asn Ile Tyr Gly Ala Leu
30                  35

AAC TGG TAT CAA CGT AAA CCT GGG AAA GCT                         210
Asn Trp Tyr Gln Arg Lys Pro Gly Lys Ala
40                  45

CCG AAG CTT CTG ATT TAC GGT GCG ACG AAC                         240
Pro Lys Leu Leu Ile Tyr Gly Ala Thr Asn
50                  55

CTG GCA GAT GGA GTC CCT TCT CGC TTC TCT                         270
Leu Ala Asp Gly Val Pro Ser Arg Phe Ser
60                  65

GGA TCC GGC TCC GGA ACG GAT TAC ACT CTG                         300
Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu
70                  75

ACC ATC AGC AGT CTG CAA CCT GAG GAC TTC                         330
Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe
80                  85

GCT ACG TAT TAC TGT CAG AAC GTT TTA AAT                         360
Ala Thr Tyr Tyr Cys Gln Asn Val Leu Asn
90                  95

ACT CCG TTG ACT TTC GGA CAG GGT ACC AAG                         390
Thr Pro Leu Thr Phe Gly Gln Gly Thr Lys
100                 105

GTG GAA ATA AAA CGA ACT GTG GCT GCA CCA                         420
Val Glu Ile Lys Arg Thr Val Ala Ala Pro
110                 115

TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG                         450
Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
120                 125

CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG                         480
Gln Leu Lys Ser Gly Thr Ala Ser Val Val
130                 135

TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG                         510
Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu
140                 145

GCC AAA GTA CAG TGG AAG GTG GAT AAC GCC                         540
Ala Lys Val Gln Trp Lys Val Asp Asn Ala
150                 155

CTC CAA TCG GGT AAC TCC CAG GAG AGT GTC                         570
Leu Gln Ser Gly Asn Ser Gln Glu Ser Val
160                 165
```

-continued

```
ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC                                  600
Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
170                 175

AGC CTC AGC AGC ACC CTG ACG CTG AGC AAA                                  630
Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys
180                 185

GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC                                  660
Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
190                 195

TGC GAA GTC ACC CAT CAG GGC CTG AGC TCG                                  690
Cys Glu Val Thr His Gln Gly Leu Ser Ser
200                 205

CCC GTC ACA AAG AGC TTC AAC AGG GGA GAG                                  720
Pro Val Thr Lys Ser Phe Asn Arg Gly Glu
210                 215

TGT TAG                                                                  726
Cys
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 726 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 VL +KLV56B
(Humanized light chain)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
ATG GGA ATC CAA GGA GGG TCT GTC CTG TTC                                   30
Met Gly Ile Gln Gly Gly Ser Val Leu Phe
-25                 -20

GGG CTG CTG CTC GTC CTG GCT GTC TTC TGC                                   60
Gly Leu Leu Leu Val Leu Ala Val Phe Cys
-15                 -10

CAT TCA GGT CAT AGC CTG CAG GAT ATC CAG                                   90
His Ser Gly His Ser Leu Gln Asp Ile Gln
-5                   1                  5

ATG ACC CAG TCC CCG TCC TCC CTG TCC GCC                                  120
Met Thr Gln Ser Pro Ser Ser Leu Ser Ala
10                  15

TCT GTG GGC GAT AGG GTC ACC ATC ACC TGC                                  150
Ser Val Gly Asp Arg Val Thr Ile Thr Cys
20                  25

GGC GCC AGC GAA AAC ATC TAT GGC GCG CTG                                  180
Gly Ala Ser Glu Asn Ile Tyr Gly Ala Leu
30                  35

AAC TGG TAT CAA CGT AAA CCT GGG AAA GCT                                  210
Asn Trp Tyr Gln Arg Lys Pro Gly Lys Ala
40                  45

CCG AAG CTT CTG ATT TAC GGT GCG ACG AAC                                  240
Pro Lys Leu Leu Ile Tyr Gly Ala Thr Asn
50                  55

CTG GCA GAT GGA GTC CCT TCT CGC TTC TCT                                  270
Leu Ala Asp Gly Val Pro Ser Arg Phe Ser
60                  65

GGA TCC GGC TCC GGA ACG GAT TTC ACT CTG                                  300
Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu
70                  75
```

-continued

```
ACC ATC AGC AGT CTG CAG CCT GAA GAC TTC                                  330
Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe
80                  85

GCT ACG TAT TAC TGT CAG AAC GTT TTA AAT                                  360
Ala Thr Tyr Tyr Cys Gln Asn Val Leu Asn
90                  95

ACT CCG TTG ACT TTC GGA CAG GGT ACC AAG                                  390
Thr Pro Leu Thr Phe Gly Gln Gly Thr Lys
100                 105

GTG GAA ATA AAA CGA ACT GTG GCT GCA CCA                                  420
Val Glu Ile Lys Arg Thr Val Ala Ala Pro
110                 115

TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG                                  450
Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
120                 125

CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG                                  480
Gln Leu Lys Ser Gly Thr Ala Ser Val Val
130                 135

TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG                                  510
Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu
140                 145

GCC AAA GTA CAG TGG AAG GTG GAT AAC GCC                                  540
Ala Lys Val Gln Trp Lys Val Asp Asn Ala
150                 155

CTC CAA TCG GGT AAC TCC CAG GAG AGT GTC                                  570
Leu Gln Ser Gly Asn Ser Gln Glu Ser Val
160                 165

ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC                                  600
Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
170                 175

AGC CTC AGC AGC ACC CTG ACG CTG AGC AAA                                  630
Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys
180                 185

GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC                                  660
Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
190                 195

TGC GAA GTC ACC CAT CAG GGC CTG AGC TCG                                  690
Cys Glu Val Thr His Gln Gly Leu Ser Ser
200                 205

CCC GTC ACA AAG AGC TTC AAC AGG GGA GAG                                  720
Pro Val Thr Lys Ser Phe Asn Arg Gly Glu
210                 215

TGT TAG                                                                  726
Cys
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 711 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 VL + O12
(Humanized light chain)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
ATG GAC ATG AGG GTC CCC GCT CAG CTC CTG                                   30
Met Asp Met Arg Val Pro Ala Gln Leu Leu
-20                 -15
```

-continued

```
GGG CTC CTG CTA CTC TGG CTC CGA GGT GCC                                  60
Gly Leu Leu Leu Leu Trp Leu Arg Gly Ala
-10                 -5

AGA TGT GAT ATC CAG ATG ACC CAG TCC CCG                                  90
Arg Cys Asp Ile Gln Met Thr Gln Ser Pro
1               5

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                                 120
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
10                  15

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                                 150
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
20                  25

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                                 180
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
30                  35

AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                                 210
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
40                  45

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                                 240
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
50                  55

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                                 270
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
60                  65

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                                 300
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
70                  75

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                                 330
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
80                  85

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                                 360
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
90                  95

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGA                                 390
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
100                 105

ACT GTG GCT GCA CCA TCT GTC TTC ATC TTC                                 420
Thr Val Ala Ala Pro Ser Val Phe Ile Phe
110                 115

CCG CCA TCT GAT GAG CAG TTG AAA TCT GGA                                 450
Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
120                 125

ACT GCC TCT GTT GTG TGC CTG CTG AAT AAC                                 480
Thr Ala Ser Val Val Cys Leu Leu Asn Asn
130                 135

TTC TAT CCC AGA GAG GCC AAA GTA CAG TGG                                 510
Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp
140                 145

AAG GTG GAT AAC GCC CTC CAA TCG GGT AAC                                 540
Lys Val Asp Asn Ala Leu Gln Ser Gly Asn
150                 155

TCC CAG GAG AGT GTC ACA GAG CAG GAC AGC                                 570
Ser Gln Glu Ser Val Thr Glu Gln Asp Ser
160                 165

AAG GAC AGC ACC TAC AGC CTC AGC AGC ACC                                 600
Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr
170                 175

CTG ACG CTG AGC AAA GCA GAC TAC GAG AAA                                 630
Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys
180                 185
```

-continued

```
CAC AAA GTC TAC GCC TGC GAA GTC ACC CAT                          660
His Lys Val Tyr Ala Cys Glu Val Thr His
190                 195

CAG GGC CTG AGC TCG CCC GTC ACA AAG AGC                          690
Gln Gly Leu Ser Ser Pro Val Thr Lys Ser
200                 205

TTC AAC AGG GGA GAG TGT TAG                                      711
Phe Asn Arg Gly Glu Cys
210
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 750 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 VH + IGHRLD
(Humanized Fd)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
ATG AAG TGG AGC TGG GTT ATT CTC TTC CTC                           30
Met Lys Trp Ser Trp Val Ile Leu Phe Leu
-15                 -10

CTG TCA GTA ACT GCC GGC GTC CAC TCC CAA                           60
Leu Ser Val Thr Ala Gly Val His Ser Gln
-5                  1

GTC CAA CTG GTG CAA TCC GGC GCC GAG GTC                           90
Val Gln Leu Val Gln Ser Gly Ala Glu Val
5                   10

AAG AAG CCA GGG GCC TCA GTC AAA GTG TCC                          120
Lys Lys Pro Gly Ala Ser Val Lys Val Ser
15                  20

TGT AAA GCT AGC GGC TAT ATT TTT TCT AAT                          150
Cys Lys Ala Ser Gly Tyr Ile Phe Ser Asn
25                  30

TAT TGG ATT CAA TGG GTG CGT CAG GCC CCC                          180
Tyr Trp Ile Gln Trp Val Arg Gln Ala Pro
35                  40

GGG CAG GGC CTG GAA TGG ATG GGT GAG ATC                          210
Gly Gln Gly Leu Glu Trp Met Gly Glu Ile
45                  50

TTA CCG GGC TCT GGT AGC ACC GAA TAT GCC                          240
Leu Pro Gly Ser Gly Ser Thr Glu Tyr Ala
55                  60

CAA AAA TTC CAG GGC CGT GTT ACT ATG ACT                          270
Gln Lys Phe Gln Gly Arg Val Thr Met Thr
65                  70

CGT GAC ACT TCG ACT AGT ACA GTA TAC ATG                          300
Arg Asp Thr Ser Thr Ser Thr Val Tyr Met
75                  80

GAG CTC TCC AGC CTG CGA TCG GAG GAC ACG                          330
Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr
85                  90

GCC GTC TAT TAT TGC GCG CGT TAT TTT TTT                          360
Ala Val Tyr Tyr Cys Ala Arg Tyr Phe Phe
95                 100

GGT TCT AGC CCG AAT TGG TAT TTT GAT GTT                          390
Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
105                 110
```

-continued

```
TGG GGT CAA GGA ACC CTG GTC ACT GTC TCG                                  420
Trp Gly Gln Gly Thr Leu Val Thr Val Ser
115                 120

AGC GCC TCC ACC AAG GGC CCA TCG GTC TTC                                  450
Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
125                 130

CCC CTG GCG CCC TCC TCC AAG AGC ACC TCT                                  480
Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
135                 140

GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC                                  510
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
145                 150

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG                                  540
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
155                 160

TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC                                  570
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
165                 170

GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC                                  600
Val His Thr Phe Pro Ala Val Leu Gln Ser
175                 180

TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG                                  630
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
185                 190

ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG                                  660
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
195                 200

ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC                                  690
Thr Tyr Ile Cys Asn Val Asn His Lys Pro
205                 210

AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG                                  720
Ser Asn Thr Lys Val Asp Lys Lys Val Glu
215                 220

CCC AAA TCT TGT GAC AAA ACT CAC ACA TAA                                  750
Pro Lys Ser Cys Asp Lys Thr His Thr
225                 230
```

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 scFv DO12
(Humanized scFv)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                                   30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                                   60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                                   90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                                  120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
35                  40
```

-continued

```
AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                                  150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                                  180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                                  210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                                  240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                                  270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                                  300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                                  330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                                  360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                                  390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                                  420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                                  450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                                  480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                                  510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                                  540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
175                 180

TCT GGT AGC ACC GAA TAT GCC CAA AAA TTC                                  570
Ser Gly Ser Thr Glu Tyr Ala Gln Lys Phe
185                 190

CAG GGC CGT GTT ACT ATG ACG CGT GAC ACT                                  600
Gln Gly Arg Val Thr Met Thr Arg Asp Thr
195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                                  630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                                  660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                                  690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                                  720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
235                 240
```

-continued

GGA ACC CTG GTC ACT GTC TCG AGC TGA 747
Gly Thr Leu Val Thr Val Ser Ser
245

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5248 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: Circular

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: pET Trc SO5/NI
prokaryotic expression vector

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TGGCGAATGG GACGCGCCCT GTAGCGGCGC ATTAAGCGCG GCGGGTGTGG 50

TGGTTACGCG CAGCGTGACC GCTACACTTG CCAGCGCCCT AGCGCCCGCT 100

CCTTTCGCTT TCTTCCCTTC CTTTCTCGCC ACGTTCGCCG GCTTTCCCCG 150

TCAAGCTCTA AATCGGGGGC TCCCTTTAGG GTTCCGATTT AGTGCTTTAC 200

GGCACCTCGA CCCCAAAAAA CTTGATTAGG GTGATGGTTC ACGTAGTGGG 250

CCATCGCCCT GATAGACGGT TTTTCGCCCT TTGACGTTGG AGTCCACGTT 300

CTTTAATAGT GGACTCTTGT TCCAAACTGG AACAACACTC AACCCTATCT 350

CGGTCTATTC TTTTGATTTA TAAGGGATTT TGCCGATTTC GGCCTATTGG 400

TTAAAAAATG AGCTGATTTA ACAAAAATTT AACGCGAATT TTAACAAAAT 450

ATTAACGTTT ACAATTTCAG GTGGCACTTT TCGGGGAAAT GTGCGCGGAA 500

CCCCTATTTG TTTATTTTTC TAAATACATT CAAATATGTA TCCGCTCATG 550

AGACAATAAC CCTGATAAAT GCTTCAATAA TATTGAAAAA GGAAGAGTAT 600

GAGTATTCAA CATTTCCGTG TCGCCCTTAT TCCCTTTTTT GCGGCATTTT 650

GCCTTCCTGT TTTTGCTCAC CCAGAAACGC TGGTGAAAGT AAAAGATGCT 700

GAAGATCAGT TGGGTGCACG AGTGGGTTAC ATCGAACTGG ATCTCAACAG 750

CGGTAAGATC CTTGAGAGTT TTCGCCCCGA AGAACGTTTT CCAATGATGA 800

GCACTTTTAA AGTTCTGCTA TGTGGCGCGG TATTATCCCG TATTGACGCC 850

GGGCAAGAGC AACTCGGTCG CCGCATACAC TATTCTCAGA ATGACTTGGT 900

TGAGTACTCA CCAGTCACAG AAAAGCATCT TACGGATGGC ATGACAGTAA 950

GAGAATTATG CAGTGCTGCC ATAACCATGA GTGATAACAC TGCGGCCAAC 1000

TTACTTCTGA CAACGATCGG AGGACCGAAG GAGCTAACCG CTTTTTTGCA 1050

CAACATGGGG GATCATGTAA CTCGCCTTGA TCGTTGGGAA CCGGAGCTGA 1100

ATGAAGCCAT ACCAAACGAC GAGCGTGACA CCACGATGCC TGCAGCAATG 1150

GCAACAACGT TGCGCAAACT ATTAACTGGC GAACTACTTA CTCTAGCTTC 1200

CCGGCAACAA TTAATAGACT GGATGGAGGC GGATAAAGTT GCAGGACCAC 1250

TTCTGCGCTC GGCCCTTCCG GCTGGCTGGT TTATTGCTGA TAAATCTGGA 1300

GCCGGTGAGC GTGGGTCTCG CGGTATCATT GCAGCACTGG GGCCAGATGG 1350

TAAGCCCTCC CGTATCGTAG TTATCTACAC GACGGGGAGT CAGGCAACTA 1400

TGGATGAACG AAATAGACAG ATCGCTGAGA TAGGTGCCTC ACTGATTAAG 1450

CATTGGTAAC TGTCAGACCA AGTTTACTCA TATATACTTT AGATTGATTT 1500

AAAACTTCAT TTTTAATTTA AAAGGATCTA GGTGAAGATC CTTTTTGATA 1550

-continued

ATCTCATGAC CAAAATCCCT TAACGTGAGT TTTCGTTCCA CTGAGCGTCA 1600

GACCCCGTAG AAAAGATCAA AGGATCTTCT TGAGATCCTT TTTTTCTGCG 1650

CGTAATCTGC TGCTTGCAAA CAAAAAAACC ACCGCTACCA GCGGTGGTTT 1700

GTTTGCCGGA TCAAGAGCTA CCAACTCTTT TTCCGAAGGT AACTGGCTTC 1750

AGCAGAGCGC AGATACCAAA TACTGTCCTT CTAGTGTAGC CGTAGTTAGG 1800

CCACCACTTC AAGAACTCTG TAGCACCGCC TACATACCTC GCTCTGCTAA 1850

TCCTGTTACC AGTGGCTGCT GCCAGTGGCG ATAAGTCGTG TCTTACCGGG 1900

TTGGACTCAA GACGATAGTT ACCGGATAAG GCGCAGCGGT CGGGCTGAAC 1950

GGGGGGTTCG TGCACACAGC CCAGCTTGGA GCGAACGACC TACACCGAAC 2000

TGAGATACCT ACAGCGTGAG CTATGAGAAA GCGCCACGCT TCCCGAAGGG 2050

AGAAAGGCGG ACAGGTATCC GGTAAGCGGC AGGGTCGGAA CAGGAGAGCG 2100

CACGAGGGAG CTTCCAGGGG GAAACGCCTG GTATCTTTAT AGTCCTGTCG 2150

GGTTTCGCCA CCTCTGACTT GAGCGTCGAT TTTTGTGATG CTCGTCAGGG 2200

GGGCGGAGCC TATGGAAAAA CGCCAGCAAC GCGGCCTTTT TACGGTTCCT 2250

GGCCTTTTGC TGGCCTTTTG CTCACATGTT CTTTCCTGCG TTATCCCCTG 2300

ATTCTGTGGA TAACCGTATT ACCGCCTTTG AGTGAGCTGA TACCGCTCGC 2350

CGCAGCCGAA CGACCGAGCG CAGCGAGTCA GTGAGCGAGG AAGCGGAAGA 2400

GCGCCTGATG CGGTATTTTC TCCTTACGCA TCTGTGCGGT ATTTCACACC 2450

GCATATATGG TGCACTCTCA GTACAATCTG CTCTGATGCC GCATAGTTAA 2500

GCCAGTATAC ACTCCGCTAT CGCTACGTGA CTGGGTCATG GCTGCGCCCC 2550

GACACCCGCC AACACCCGCT GACGCGCCCT GACGGGCTTG TCTGCTCCCG 2600

GCATCCGCTT ACAGACAAGC TGTGACCGTC TCCGGGAGCT GCATGTGTCA 2650

GAGGTTTTCA CCGTCATCAC CGAAACGCGC GAGGCAGCTG CGGTAAAGCT 2700

CATCAGCGTG GTCGTGAAGC GATTCACAGA TGTCTGCCTG TTCATCCGCG 2750

TCCAGCTCGT TGAGTTTCTC CAGAAGCGTT AATGTCTGGC TTCTGATAAA 2800

GCGGGCCATG TTAAGGGCGG TTTTTTCCTG TTTGGTCACT GATGCCTCCG 2850

TGTAAGGGGG ATTTCTGTTC ATGGGGGTAA TGATACCGAT GAAACGAGAG 2900

AGGATGCTCA CGATACGGGT TACTGATGAT GAACATGCCC GGTTACTGGA 2950

ACGTTGTGAG GGTAAACAAC TGGCGGTATG GATGCGGCGG GACCAGAGAA 3000

AAATCACTCA GGGTCAATGC CAGCGCTTCG TTAATACAGA TGTAGGTGTT 3050

CCACAGGGTA GCCAGCAGCA TCCTGCGATG CAGATCCGGA ACATAATGGT 3100

GCAGGGCGCT GACTTCCGCG TTTCCAGACT TTACGAAACA CGGAAACCGA 3150

AGACCATTCA TGTTGTTGCT CAGGTCGCAG ACGTTTTGCA GCAGCAGTCG 3200

CTTCACGTTC GCTCGCGTAT CGGTGATTCA TTCTGCTAAC CAGTAAGGCA 3250

ACCCCGCCAG CCTAGCCGGG TCCTCAACGA CAGGAGCACG ATCATGCGCA 3300

CCCGTGGGGC CGCCATGCCG GCGATAATGG CCTGCTTCTC GCCGAAACGT 3350

TTGGTGGCGG GACCAGTGAC GAAGGCTTGA GCGAGGGCGT GCAAGATTCC 3400

GAATACCGCA AGCGACAGGC CGATCATCGT CGCGCTCCAG CGAAAGCGGT 3450

CCTCGCCGAA AATGACCCAG AGCGCTGCCG GCACCTGTCC TACGAGTTGC 3500

ATGATAAAGA AGACAGTCAT AAGTGCGGCG ACGATAGTCA TGCCCCGCGC 3550

-continued

CCACCGGAAG GAGCTGACTG GGTTGAAGGC TCTCAAGGGC ATCGGTCGAG 3600

ATCCCGGTGC CTAATGAGTG AGCTAACTTA CATTAATTGC GTTGCGCTCA 3650

CTGCCCGCTT TCCAGTCGGG AAACCTGTCG TGCCAGCTGC ATTAATGAAT 3700

CGGCCAACGC GCGGGGAGAG GCGGTTTGCG TATTGGGCGC CAGGGTGGTT 3750

TTTCTTTTCA CCAGTGAGAC GGGCAACAGC TGATTGCCCT TCACCGCCTG 3800

GCCCTGAGAG AGTTGCAGCA AGCGGTCCAC GCTGGTTTGC CCCAGCAGGC 3850

GAAAATCCTG TTTGATGGTG GTTAACGGCG GGATATAACA TGAGCTGTCT 3900

TCGGTATCGT CGTATCCCAC TACCGAGATA TCCGCACCAA CGCGCAGCCC 3950

GGACTCGGTA ATGGCGCGCA TTGCGCCCAG CGCCATCTGA TCGTTGGCAA 4000

CCAGCATCGC AGTGGGAACG ATGCCCTCAT TCAGCATTTG CATGGTTTGT 4050

TGAAAACCGG ACATGGCACT CCAGTCGCCT TCCCGTTCCG CTATCGGCTG 4100

AATTTGATTG CGAGTGAGAT ATTTATGCCA GCCAGCCAGA CGCAGACGCG 4150

CCGAGACAGA ACTTAATGGG CCCGCTAACA GCGCGATTTG CTGGTGACCC 4200

AATGCGACCA GATGCTCCAC GCCCAGTCGC GTACCGTCTT CATGGGAGAA 4250

AATAATACTG TTGATGGGTG TCTGGTCAGA GACATCAAGA AATAACGCCG 4300

GAACATTAGT GCAGGCAGCT TCCACAGCAA TGGCATCCTG GTCATCCAGC 4350

GGATAGTTAA TGATCAGCCC ACTGACGCGT TGCGCGAGAA GATTGTGCAC 4400

CGCCGCTTTA CAGGCTTCGA CGCCGCTTCG TTCTACCATC GACACCACCA 4450

CGCTGGCACC CAGTTGATCG GCGCGAGATT TAATCGCCGC GACAATTTGC 4500

GACGGCGCGT GCAGGGCCAG ACTGGAGGTG GCAACGCCAA TCAGCAACGA 4550

CTGTTTGCCC GCCAGTTGTT GTGCCACGCG GTTGGGAATG TAATTCAGCT 4600

CCGCCATCGC CGCTTCCACT TTTTCCCGCG TTTTCGCAGA AACGTGGCTG 4650

GCCTGGTTCA CCACGCGGGA AACGGTCTGA TAAGAGACAC CGGCATACTC 4700

TGCGACATCG TATAACGTTA CTGGTTTCAC ATTCACCACC CTGAATTGAC 4750

TCTCTTCCGG GCGCTATCAT GCCATACCGC GAAAGGTTTT GCGCCATTCG 4800

ATGGTGTCCG GGATCTCGAC GCTCTCCCTT ATGCGACTCC TGCATTAGGA 4850

AGCAGCCCAG TAGTAGGTTG AGGCCGTTGA GCACCGCCGC CGCAAGGAAT 4900

GGTGCATGCG GTACCAGCTG TTGACAATTA ATCATCCGGC TCGTATAATA 4950

GTACTGTGTG GAATTGTGAG CGCTCACAAT TCCACACATC TAGAAATAAT 5000

TTTGTTTAAC TTTAAGAAGG AGATATACCA TGGAGATCTG GATCCATCGA 5050

TGAATTCGAG CTCCGTCGAC AAGCTTGCGG CCGCACTCGA GCACCACCAC 5100

CACCACCACT GAGATCCGGC TGCTAACAAA GCCCGAAAGG AAGCTGAGTT 5150

GGCTGCTGCC ACCGCTGAGC AATAACTAGC ATAACCCCTT GGGGCCTCTA 5200

AACGGGTCTT GAGGGGTTTT TTGCTGAAAG GAGGAACTAT ATCCGGAT 5248

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 783 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: N19/8 scFv (His Tagged)

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```
ATG GCC AAT ATT GTG CTG ACC CAA TCT CCA                                  30
Met Ala Asn Ile Val Leu Thr Gln Ser Pro
1               5                   10

GCT TCT TTG GCT GTG TCT CTA GGG CAG AGG                                  60
Ala Ser Leu Ala Val Ser Leu Gly Gln Arg
15                  20

GCC ACC ATA TCC TGC AGA GCC AGT GAA AGT                                  90
Ala Thr Ile Ser Cys Arg Ala Ser Glu Ser
25                  30

GTT GAT AGT TAT GAC AAT AGT TTT ATG CAC                                 120
Val Asp Ser Tyr Asp Asn Ser Phe Met His
35                  40

TGG TAC CAG CAG AAA CCA GGA CAG CCA CCC                                 150
Trp Tyr Gln Gln Lys Pro Gly Gln Pro Pro
45                  50

AAA CTC CTC ATC TTT CTT GCA TCC AAC CTA                                 180
Lys Leu Leu Ile Phe Leu Ala Ser Asn Leu
55                  60

GAA TCT GGG GTC CCT GCC AGG TTC AGT GGC                                 210
Glu Ser Gly Val Pro Ala Arg Phe Ser Gly
65                  70

AGT GGG TCT AGG ACA GAC TTC ACC CTC ACC                                 240
Ser Gly Ser Arg Thr Asp Phe Thr Leu Thr
75                  80

ATT GAT CCT GTG GAG GCT GAT GAT GCT GCA                                 270
Ile Asp Pro Val Glu Ala Asp Asp Ala Ala
85                  90

ACC TAT TAC TGT CAG CAA AAT AAT GAG GTT                                 300
Thr Tyr Tyr Cys Gln Gln Asn Asn Glu Val
95                 100

CCG AAC ACG TTC GGA GGG GGG ACC AAG CTG                                 330
Pro Asn Thr Phe Gly Gly Gly Thr Lys Leu
105                 110

GAA ATA AAA CGG ACC GGA GGT GGC GGG TCG                                 360
Glu Ile Lys Arg Thr Gly Gly Gly Gly Ser
115                 120

GGT GGC GGG GGA TCG GGT GGC GGA GGG TCG                                 390
Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
125                 130

GAC GTC AAG CTC GTG GAG TCT GGG GGA GAC                                 420
Asp Val Lys Leu Val Glu Ser Gly Gly Asp
135                 140

TTA GTG AAG CTT GGA GGG TCC CTG AAA CTC                                 450
Leu Val Lys Leu Gly Gly Ser Leu Lys Leu
145                 150

TCC TGT GCA GCC TCT GGA TTC ACC TTC AGT                                 480
Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
155                 160

AGC TAT TAT ATG TCT TGG GTT CGC CAG ATT                                 510
Ser Tyr Tyr Met Ser Trp Val Arg Gln Ile
165                 170

TCA GAG AAG AGG CTG GAG TTG GTC GCA GCC                                 540
Ser Glu Lys Arg Leu Glu Leu Val Ala Ala
175                 180

ATT AAT AGT AAT GGT GAT AGC ACC TAC TAT                                 570
Ile Asn Ser Asn Gly Asp Ser Thr Tyr Tyr
185                 190
```

-continued

```
CCA GAC ACT GTG AAG GGC CGA TTC ACC ATC                                  600
Pro Asp Thr Val Lys Gly Arg Phe Thr Ile
195                 200

TCC AGA GAC AAT GCC AAG AGC ACC CTG GAT                                  630
Ser Arg Asp Asn Ala Lys Ser Thr Leu Asp
205                 210

CTG CAA ATG AGC AGT CTG AAG TCT GAG GAC                                  660
Leu Gln Met Ser Ser Leu Lys Ser Glu Asp
215                 220

ACA GCC TTG TAT TTC TGT GTA AGA GAG ACT                                  690
Thr Ala Leu Tyr Phe Cys Val Arg Glu Thr
225                 230

TAT TAC TAC GGG ATT AGT CCC GTC TTC GAT                                  720
Tyr Tyr Tyr Gly Ile Ser Pro Val Phe Asp
235                 240

GTC TGG GGC ACA GGG ACC ACG GTC ACC GTC                                  750
Val Trp Gly Thr Gly Thr Thr Val Thr Val
245                 250

TCC TCA CTC GAG CAC CAC CAC CAC CAC CAC                                  780
Ser Ser Leu Glu His His His His His His
255                 260

TGA                                                                      783
```

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 scFv C012 (humanized)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                                   30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                                   60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                                   90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                                  120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
35                  40

AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                                  150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                                  180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                                  210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                                  240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                                  270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
85                  90
```

-continued

```
CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                                  300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                                  330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                                  360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                                  390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                                  420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                                  450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                                  480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                                  510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                                  540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                                  570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                                  600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                                  630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                                  660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                                  690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                                  720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                                      747
Gly Thr Leu Val Thr Val Ser Ser
245
```

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 scFv DO12B
(Humanized scFv)

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                                  30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                                  60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
15                  20

GTC ACC ATC ACC TGC CGT GCT AGC GAA AAC                                  90
Val Thr Ile Thr Cys Arg Ala Ser Glu Asn
25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                                 120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
35                  40

AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                                 150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                                 180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                                 210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                                 240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                                 270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                                 300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                                 330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                                 360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                                 390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                                 420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                                 450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                                 480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                                 510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                                 540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
175                 180

TCT GGT AGC ACC GAA TAT GCC CAA AAA TTC                                 570
Ser Gly Ser Thr Glu Tyr Ala Gln Lys Phe
185                 190
```

-continued

```
CAG GGC CGT GTT ACT ATG ACG CGT GAC ACT                          600
Gln Gly Arg Val Thr Met Thr Arg Asp Thr
195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                          630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                          660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                          690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                          720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                              747
Gly Thr Leu Val Thr Val Ser Ser
245

(2) INFORMATION FOR SEQ ID NO:22:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH:  747 base pairs
          (B) TYPE:  Nucleic Acid
          (C) STRANDEDNESS:  Double
          (D) TOPOLOGY:  linear

    (ii) MOLECULE TYPE:  Other nucleic acid
          (A) DESCRIPTION:5G1.1 scFv DO12C
          (Humanized scFv)

    (xi) SEQUENCE DESCRIPTION:  SEQ ID NO:22:

ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                           30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                           60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                           90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                          120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
35                  40

AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                          150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
45                  50

TAC GGT GCG ACG AGC CTG CAG TCT GGA GTC                          180
Tyr Gly Ala Thr Ser Leu Gln Ser Gly Val
55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                          210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                          240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                          270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                          300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                 100
```

-continued

```
GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                                  330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                                  360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                                  390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                                  420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                                  450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                                  480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                                  510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                                  540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
175                 180

TCT GGT AGC ACC GAA TAT GCC CAA AAA TTC                                  570
Ser Gly Ser Thr Glu Tyr Ala Gln Lys Phe
185                 190

CAG GGC CGT GTT ACT ATG ACG CGT GAC ACT                                  600
Gln Gly Arg Val Thr Met Thr Arg Asp Thr
195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                                  630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                                  660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                                  690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                                  720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                                      747
Gly Thr Leu Val Thr Val Ser Ser
245
```

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 scFv DO12D
(Humanized scFv)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                                   30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10
```

-continued

```
TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                                  60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
15                  20

GTC ACC ATC ACC TGC CGT GCT AGC GAA AAC                                  90
Val Thr Ile Thr Cys Arg Ala Ser Glu Asn
25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                                 120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
35                  40

AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                                 150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
45                  50

TAC GGT GCG ACG AGC CTG CAG TCT GGA GTC                                 180
Tyr Gly Ala Thr Ser Leu Gln Ser Gly Val
55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                                 210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                                 240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                                 270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                                 300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                                 330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                                 360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                                 390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                                 420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                                 450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                                 480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                                 510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                                 540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
175                 180

TCT GGT AGC ACC GAA TAT GCC CAA AAA TTC                                 570
Ser Gly Ser Thr Glu Tyr Ala Gln Lys Phe
185                 190

CAG GGC CGT GTT ACT ATG ACG CGT GAC ACT                                 600
Gln Gly Arg Val Thr Met Thr Arg Asp Thr
195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                                 630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
205                 210
```

-continued

```
AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                                  660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                                  690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                                  720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                                      747
Gly Thr Leu Val Thr Val Ser Ser
245
```

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 scFv C013 (humanized)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                                   30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                                   60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
15                  20

GTC ACC ATC ACC TGC CGT GCT AGC GAA AAC                                   90
Val Thr Ile Thr Cys Arg Ala Ser Glu Asn
25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                                  120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
35                  40

AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                                  150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                                  180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                                  210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                                  240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                                  270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                                  300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                                  330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                                  360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120
```

-continued

```
TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                                  390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                                  420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                                  450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                                  480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                                  510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                                  540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                                  570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                                  600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                                  630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                                  660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                                  690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                                  720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                                      747
Gly Thr Leu Val Thr Val Ser Ser
245
```

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 scFv C014 (humanized)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                                   30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                                   60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                                   90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
25                  30
```

-continued

```
ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                                  120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
35                  40

AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                                  150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
45                  50

TAC GGT GCG ACG AGC CTG CAG TCT GGA GTC                                  180
Tyr Gly Ala Thr Ser Leu Gln Ser Gly Val
55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                                  210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                                  240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                                  270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                                  300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                                  330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                                  360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                                  390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                                  420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                                  450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                                  480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                                  510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                                  540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                                  570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                                  600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                                  630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                                  660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                                  690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230
```

-continued

```
CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                                  720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                                      747
Gly Thr Leu Val Thr Val Ser Ser
245
```

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 scFv C015 (humanized)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                                   30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                                   60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
15                  20

GTC ACC ATC ACC TGC CGT GCT AGC GAA AAC                                   90
Val Thr Ile Thr Cys Arg Ala Ser Glu Asn
25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                                  120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
35                  40

AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                                  150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
45                  50

TAC GGT GCG ACG AGC CTG CAG TCT GGA GTC                                  180
Tyr Gly Ala Thr Ser Leu Gln Ser Gly Val
55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                                  210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                                  240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                                  270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                                  300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                                  330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                                  360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                                  390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                                  420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
135                 140
```

-continued

```
GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                    570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                        747
Gly Thr Leu Val Thr Val Ser Ser
245
```

What is claimed is:

1. An antibody comprising at least one antibody-antigen binding site, said antibody exhibiting specific binding to human complement component C5, said specific binding being targeted to the alpha chain of human complement component C5, wherein the antibody 1) inhibits complement activation in a human body fluid, 2) inhibits the binding of purified human complement component C5 to either human complement component C3 or human complement component C4, and 3) does not specifically bind to the human complement activation product free C5a.

2. The antibody of claim **1** wherein the inhibition of complement activation in the human body fluid is measurable as an increment of blockade of C5a generation and an increment of blockade of complement hemolytic activity in the body fluid, said increment of blockade of C5a generation being substantially equal to said increment of blockade of complement hemolytic activity.

3. The antibody of claim **1** wherein, upon binding to human C5, there is a 60% to 90% reduction in the ability of C5 to bind to human complement component C3.

4. The antibody of claim **1** wherein, upon binding to human C5, there is a 60% to 90% reduction in the ability of C5 to bind to human complement component C4.

5. The antibody of claim **1** wherein the antibody binds specifically to an isolated oligopeptide comprising an amino acid sequence corresponding to amino acid 8 through amino acid 12 of SEQ ID NO:1.

6. The antibody of claim **1** wherein the inhibition of complement activation in the human body fluid is measurable as a substantially complete blockade of C5a generation in the body fluid and a substantially complete blockade of complement hemolytic activity in the body fluid when the antibody is added to the body fluid at a concentration yielding a ratio equal to or less than 10 moles of antibody-antigen binding sites of the antibody to 1 mole of human C5 in the body fluid.

7. The antibody of claim **1** wherein the antibody is a humanized antibody.

8. The antibody of claim **1** wherein the antibody is an scFv.

9. The antibody of claim **1**, wherein, when administered to a human patient via intravenous infusion, the antibody provides complete complement inhibition at dosages below 0.005 g/kg.

10. The antibody of claim **1**, wherein, when administered to a human patient via intravenous infusion, the antibody provides therapeutic benefits at dosages below 0.0022 g/kg.

11. The antibody of claim **10**, wherein the antibody is administered in association with an extracorporeal circulation procedure.

12. The antibody of claim **1** wherein the inhibition of complement activation in the human body fluid is measurable as a substantially complete blockade of C5a generation in the body fluid and a substantially complete blockade of complement hemolytic activity in the body fluid when the

antibody is added to the body fluid at a concentration yielding a ratio equal to or less than 3 moles of antibody-antigen binding sites of the antibody to 1 mole of human C5 in the body fluid.

13. The antibody of claim **1**, wherein, when administered to a human patient via intravenous infusion, the antibody provides therapeutically effective complement inhibition at dosages below 0.003 g/kg.

14. A sterile non-pyrogenic therapeutic agent comprising the antibody of claim **1** in a formulation suitable for administration to a human.

15. The therapeutic agent of claim **14** wherein the antibody is a humanized immunoglobulin.

16. The therapeutic agent of claim **14** wherein the antibody is an scFv.

17. The therapeutic agent of claim **14** wherein the antibody is made up of two or more heterodimeric subunits each containing one heavy and one light chain.

18. Antibody 5G1.1 scFv CB (humanized) having the amino acid sequence encoded by the nucleic acid sequence of SEQ ID NO:8.

19. An isolated antigen binding protein comprising:

1) a variable light region CDR1 comprising an amino acid sequence corresponding to amino acid residues 26–36 of SEQ ID NO:8,

2) a variable light region CDR2 comprising an amino acid sequence corresponding to amino acid residues 52–58 of SEQ ID NO:8,

3) a variable light region CDR3 comprising an amino acid sequence corresponding to amino acid residues 91 through amino acid 99 of SEQ ID NO:8,

4) a variable heavy region CDR1 comprising an amino acid sequence corresponding to amino acid residues 152 through amino acid 161 of SEQ ID NO:8,

5) a variable heavy region CDR2 comprising an amino acid sequence corresponding to amino acid residues 176 through amino acid 192 of SEQ ID NO:8,

6) a variable heavy region CDR3 comprising an amino acid sequence corresponding to amino acid residues 225 through amino acid 237 of SEQ ID NO:8,

said protein exhibiting specific binding to human complement component C5, said specific binding being targeted to the alpha chain of human complement component C5, wherein the protein inhibits complement activation in a human body fluid and does not specifically bind to the human complement activation product free C5a.

20. A nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO: 12.

21. Hybridoma 5G1.1 having ATCC designation HB-11625.

22. An antibody produced by the hybridoma of claim **21**.

23. An antibody comprising at least one antibody-antigen binding site, said antibody exhibiting specific binding to human complement component C5, said specific binding being targeted to the alpha chain of human complement component C5, wherein:

(A) the antibody inhibits (i) C5b-9-mediated hemolysis and (ii) C5a generation in a fluid comprising human serum; and

(B) the antibody does not specifically bind to the human complement activation product free C5a.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page, and Column 1, lines 1-3,
Delete the title "**C5-SPECIFIC ANTIBODIES FOR THE TREATMENT OF INFLAMMATORY DISEASES**" and insert therefor -- **ANTIBODIES TO HUMAN COMPLEMENT COMPONENT C5** --.

Column 19,
Line 54, delete "(SEQ ID NO:1)".
Line 55, following "KSSKC peptide", insert -- (SEQ ID NO:1) --.

Column 55,
Please delete lines 50-53, and insert therefor:

```
Val Ile Asp His Gln Gly Thr Lys Ser Ser
                 5                  10

Lys Cys Val Arg Gln Lys Val Glu Gly Ser Ser
                15                  20
```

Column 57,
Please delete lines 22-37, and insert therefor:

```
                  Met Gly Leu Leu Gly Ile Leu Cys Phe Leu
                             -15                 -10

  Ile Phe Leu Gly Lys Thr Trp Gly Gln Glu Gln Thr Tyr Val
              -5              -1                   5

  Ile Ser Ala Pro Lys Ile Phe Arg Val Gly Ala Ser Glu Asn
             10                  15                  20

  Ile Val Ile Gln Val Tyr Gly Tyr Thr Glu Ala Phe Asp Ala
                  25                  30

  Thr Ile Ser Ile Lys Ser Tyr Pro Asp Lys Lys Phe Ser Tyr
   35                 40                  45

  Ser Ser Gly His Val His Leu Ser Ser Glu Asn Lys Phe Gln
       50                 55                  60

  Asn Ser Ala Ile Leu Thr Ile Gln Pro Lys Gln Leu Pro Gly
            65                 70                  75

  Gly Gln Asn Pro Val Ser Tyr Val Tyr Leu Glu Val Val Ser
             80                 85                  90
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 57,
Please delete lines 38-55, and insert therefor:

```
Lys His Phe Ser Lys Ser Lys Arg Met Pro Ile Thr Tyr Asp
                95                  100

Asn Gly Phe Leu Phe Ile His Thr Asp Lys Pro Val Tyr Thr
105                 110                 115

Pro Asp Gln Ser Val Lys Val Arg Val Tyr Ser Leu Asn Asp
    120                 125                 130

Asp Leu Lys Pro Ala Lys Arg Glu Thr Val Leu Thr Phe Ile
        135                 140                 145

Asp Pro Glu Gly Ser Glu Val Asp Met Val Glu Glu Ile Asp
            150                 155                 160

His Ile Gly Ile Ile Ser Phe Pro Asp Phe Lys Ile Pro Ser
                165                 170

Asn Pro Arg Tyr Gly Met Trp Thr Ile Lys Ala Lys Tyr Lys
175                 180                 185

Glu Asp Phe Ser Thr Thr Gly Thr Ala Tyr Phe Glu Val Lys
    190                 195                 200

Glu Tyr Val Leu Pro His Phe Ser Val Ser Ile Glu Pro Glu
        205                 210                 215
```

Column 59,
Please delete lines 1-4, and insert therefor:

```
Tyr Asn Phe Ile Gly Tyr Lys Asn Phe Lys Asn Phe Glu Ile
            220                 225                 230

Thr Ile Lys Ala Arg Tyr Phe Tyr Asn Lys Val Val Thr Glu
                235                 240
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 59,
Please delete lines 5-28, and insert therefor:

```
Ala Asp Val Tyr Ile Thr Phe Gly Ile Arg Glu Asp Leu Lys
245                 250                 255

Asp Asp Gln Lys Glu Met Met Gln Thr Ala Met Gln Asn Thr
    260                 265                 270

Met Leu Ile Asn Gly Ile Ala Gln Val Thr Phe Asp Ser Glu
        275                 280                 285

Thr Ala Val Lys Glu Leu Ser Tyr Tyr Ser Leu Glu Asp Leu
            290                 295                 300

Asn Asn Lys Tyr Leu Tyr Ile Ala Val Thr Val Ile Glu Ser
                305                 310

Thr Gly Gly Phe Ser Glu Glu Ala Glu Ile Pro Gly Ile Lys
315                 320                 325

Tyr Val Leu Ser Pro Tyr Lys Leu Asn Leu Val Ala Thr Pro
    330                 335                 340

Leu Phe Leu Lys Pro Gly Ile Pro Tyr Pro Ile Lys Val Gln
        345                 350                 355

Val Lys Asp Ser Leu Asp Gln Leu Val Gly Gly Val Pro Val
            360                 365                 370

Ile Leu Asn Ala Gln Thr Ile Asp Val Asn Gln Glu Thr Ser
                375                 380

Asp Leu Asp Pro Ser Lys Ser Val Thr Arg Val Asp Asp Gly
385                 390                 395

Val Ala Ser Phe Val Leu Asn Leu Pro Ser Gly Val Thr Val
    400                 405                 410
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 59,
Please delete lines 29-52, and insert therefor:

```
Leu Glu Phe Asn Val Lys Thr Asp Ala Pro Asp Leu Pro Glu
        415                 420                 425

Glu Asn Gln Ala Arg Glu Gly Tyr Arg Ala Ile Ala Tyr Ser
            430                 435                 440

Ser Leu Ser Gln Ser Tyr Leu Tyr Ile Asp Trp Thr Asp Asn
                445                 450

His Lys Ala Leu Leu Val Gly Glu His Leu Asn Ile Ile Val
455                 460                 465

Thr Pro Lys Ser Pro Tyr Ile Asp Lys Ile Thr His Tyr Asn
    470                 475                 480

Tyr Leu Ile Leu Ser Lys Gly Lys Ile Ile His Phe Gly Thr
        485                 490                 495

Arg Glu Lys Phe Ser Asp Ala Ser Tyr Gln Ser Ile Asn Ile
            500                 505                 510

Pro Val Thr Gln Asn Met Val Pro Ser Ser Arg Leu Leu Val
                515                 520

Tyr Tyr Ile Val Thr Gly Glu Gln Thr Ala Glu Leu Val Ser
525                 530                 535

Asp Ser Val Trp Leu Asn Ile Glu Glu Lys Cys Gly Asn Gln
    540                 545                 550

Leu Gln Val His Leu Ser Pro Asp Ala Asp Ala Tyr Ser Pro
        555                 560                 565

Gly Gln Thr Val Ser Leu Asn Met Ala Thr Gly Met Asp Ser
            570                 575                 580
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 61,
Please delete lines 1-26, and insert therefor:

```
Trp Val Ala Leu Ala Ala Val Asp Ser Ala Val Tyr Gly Val
                585                 590

Gln Arg Gly Ala Lys Lys Pro Leu Glu Arg Val Phe Gln Phe
595                 600                 605

Leu Glu Lys Ser Asp Leu Gly Cys Gly Ala Gly Gly Gly Leu
    610                 615                 620

Asn Asn Ala Asn Val Phe His Leu Ala Gly Leu Thr Phe Leu
        625                 630                 635

Thr Asn Ala Asn Ala Asp Asp Ser Gln Glu Asn Asp Glu Pro
            640                 645                 650

Cys Lys Glu Ile Leu Arg Pro Arg Arg Thr Leu Gln Lys Lys
                655                 660

Ile Glu Glu Ile Ala Ala Lys Tyr Lys His Ser Val Val Lys
665                 670                 675

Lys Cys Cys Tyr Asp Gly Ala Cys Val Asn Asn Asp Glu Thr
    680                 685                 690

Cys Glu Gln Arg Ala Ala Arg Ile Ser Leu Gly Pro Arg Cys
        695                 700                 705

Ile Lys Ala Phe Thr Glu Cys Cys Val Val Ala Ser Gln Leu
            710                 715                 720

Arg Ala Asn Ile Ser His Lys Asp Met Gln Leu Gly Arg Leu
                725                 730

His Met Lys Thr Leu Leu Pro Val Ser Lys Pro Glu Ile Arg
735                 740                 745

Ser Tyr Phe Pro Glu Ser Trp Leu Trp Glu Val His Leu Val
    750                 755                 760
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 61,
Please delete lines 27-52, and insert therefor:

```
Pro Arg Arg Lys Gln Leu Gln Phe Ala Leu Pro Asp Ser Leu
        765                 770                 775

Thr Thr Trp Glu Ile Gln Gly Ile Gly Ile Ser Asn Thr Gly
            780                 785                 790

Ile Cys Val Ala Asp Thr Val Lys Ala Lys Val Phe Lys Asp
                795                 800

Val Phe Leu Glu Met Asn Ile Pro Tyr Ser Val Val Arg Gly
805                 810                 815

Glu Gln Ile Gln Leu Lys Gly Thr Val Tyr Asn Tyr Arg Thr
    820                 825                 830

Ser Gly Met Gln Phe Cys Val Lys Met Ser Ala Val Glu Gly
        835                 840                 845

Ile Cys Thr Ser Glu Ser Pro Val Ile Asp His Gln Gly Thr
            850                 855                 860

Lys Ser Ser Lys Cys Val Arg Gln Lys Val Glu Gly Ser Ser
                865                 870

Ser His Leu Val Thr Phe Thr Val Leu Pro Leu Glu Ile Gly
875                 880                 885

Leu His Asn Ile Asn Phe Ser Leu Glu Thr Trp Phe Gly Lys
    890                 895                 900

Glu Ile Leu Val Lys Thr Leu Arg Val Val Pro Glu Gly Val
        905                 910                 915

Lys Arg Glu Ser Tyr Ser Gly Val Thr Leu Asp Pro Arg Gly
            920                 925                 930

Ile Tyr Gly Thr Ile Ser Arg Arg Lys Glu Phe Pro Tyr Arg
                935                 940
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 63,
Please delete lines 1-26, and insert therefor:

```
Ile Pro Leu Asp Leu Val Pro Lys Thr Glu Ile Lys Arg Ile
945                 950                 955

Leu Ser Val Lys Gly Leu Leu Val Gly Glu Ile Leu Ser Ala
    960                 965                 970

Val Leu Ser Gln Glu Gly Ile Asn Ile Leu Thr His Leu Pro
        975                 980                 985

Lys Gly Ser Ala Glu Ala Glu Leu Met Ser Val Val Pro Val
            990                 995                 1000

Phe Tyr Val Phe His Tyr Leu Glu Thr Gly Asn His Trp Asn
                1005                1010

Ile Phe His Ser Asp Pro Leu Ile Glu Lys Gln Lys Leu Lys
1015                1020                1025

Lys Lys Leu Lys Glu Gly Met Leu Ser Ile Met Ser Tyr Arg
    1030                1035                1040

Asn Ala Asp Tyr Ser Tyr Ser Val Trp Lys Gly Gly Ser Ala
        1045                1050                1055

Ser Thr Trp Leu Thr Ala Phe Ala Leu Arg Val Leu Gly Gln
            1060                1065                1070

Val Asn Lys Tyr Val Glu Gln Asn Gln Asn Ser Ile Cys Asn
                1075                1080

Ser Leu Leu Trp Leu Val Glu Asn Tyr Gln Leu Asp Asn Gly
1085                1090                1095

Ser Phe Lys Glu Asn Ser Gln Tyr Gln Pro Ile Lys Leu Gln
    1100                1105                1110

Gly Thr Leu Pro Val Glu Ala Arg Glu Asn Ser Leu Tyr Leu
        1115                1120                1125
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 63,
Please delete lines 27-52, and insert therefor:

```
Thr Ala Phe Thr Val Ile Gly Ile Arg Lys Ala Phe Asp Ile
            1130                1135                1140

Cys Pro Leu Val Lys Ile Asp Thr Ala Leu Ile Lys Ala Asp
                1145                1150

Asn Phe Leu Leu Glu Asn Thr Leu Pro Ala Gln Ser Thr Phe
1155                1160                1165

Thr Leu Ala Ile Ser Ala Tyr Ala Leu Ser Leu Gly Asp Lys
    1170                1175                1180

Thr His Pro Gln Phe Arg Ser Ile Val Ser Ala Leu Lys Arg
        1185                1190                1195

Glu Ala Leu Val Lys Gly Asn Pro Pro Ile Tyr Arg Phe Trp
            1200                1205                1210

Lys Asp Asn Leu Gln His Lys Asp Ser Ser Val Pro Asn Thr
                1215                1220

Gly Thr Ala Arg Met Val Glu Thr Thr Ala Tyr Ala Leu Leu
1225                1230                1235

Thr Ser Leu Asn Leu Lys Asp Ile Asn Tyr Val Asn Pro Val
    1240                1245                1250

Ile Lys Trp Leu Ser Glu Glu Gln Arg Tyr Gly Gly Gly Phe
        1255                1260                1265

Tyr Ser Thr Gln Asp Thr Ile Asn Ala Ile Glu Gly Leu Thr
            1270                1275                1280

Glu Tyr Ser Leu Leu Val Lys Gln Leu Arg Leu Ser Met Asp
                1285                1290

Ile Asp Val Ser Tyr Lys His Lys Gly Ala Leu His Asn Tyr
1295                1300                1305
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 65,
Please delete lines 1-26, and insert therefor:

```
Lys Met Thr Asp Lys Asn Phe Leu Gly Arg Pro Val Glu Val
    1310                1315                1320

Leu Leu Asn Asp Asp Leu Ile Val Ser Thr Gly Phe Gly Ser
        1325                1330                1335

Gly Leu Ala Thr Val His Val Thr Thr Val Val His Lys Thr
            1340                1345                1350

Ser Thr Ser Glu Glu Val Cys Ser Phe Tyr Leu Lys Ile Asp
                1355                1360

Thr Gln Asp Ile Glu Ala Ser His Tyr Arg Gly Tyr Gly Asn
1365                1370                1375

Ser Asp Tyr Lys Arg Ile Val Ala Cys Ala Ser Tyr Lys Pro
    1380                1385                1390

Ser Arg Glu Glu Ser Ser Ser Gly Ser Ser His Ala Val Met
        1395                1400                1405

Asp Ile Ser Leu Pro Thr Gly Ile Ser Ala Asn Glu Glu Asp
            1410                1415                1420

Leu Lys Ala Leu Val Glu Gly Val Asp Gln Leu Phe Thr Asp
                1425                1430

Tyr Gln Ile Lys Asp Gly His Val Ile Leu Gln Leu Asn Ser
1435                1440                1445

Ile Pro Ser Ser Asp Phe Leu Cys Val Arg Phe Arg Ile Phe
    1450                1455                1460

Glu Leu Phe Glu Val Gly Phe Leu Ser Pro Ala Thr Phe Thr
        1465                1470                1475

Val Tyr Glu Tyr His Arg Pro Asp Lys Gln Cys Thr Met Phe
            1480                1485                1490
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 65,
Please delete lines 27-50, and insert therefor:

```
Tyr Ser Thr Ser Asn Ile Lys Ile Gln Lys Val Cys Glu Gly
                1495                1500

Ala Ala Cys Lys Cys Val Glu Ala Asp Cys Gly Gln Met Gln
1505                1510                1515

Glu Glu Leu Asp Leu Thr Ile Ser Ala Glu Thr Arg Lys Gln
    1520                1525                1530

Thr Ala Cys Lys Pro Glu Ile Ala Tyr Ala Tyr Lys Val Ser
        1535                1540                1545

Ile Thr Ser Ile Thr Val Glu Asn Val Phe Val Lys Tyr Lys
            1550                1555                1560

Ala Thr Leu Leu Asp Ile Tyr Lys Thr Gly Glu Ala Val Ala
                1565                1570

Glu Lys Asp Ser Glu Ile Thr Phe Ile Lys Lys Val Thr Cys
1575                1580                1585

Thr Asn Ala Glu Leu Val Lys Gly Arg Gln Tyr Leu Ile Met
    1590                1595                1600

Gly Lys Glu Ala Leu Gln Ile Lys Tyr Asn Phe Ser Phe Arg
        1605                1610                1615

Tyr Ile Tyr Pro Leu Asp Ser Leu Thr Trp Ile Glu Tyr Trp
            1620                1625                1630

Pro Arg Asp Thr Thr Cys Ser Ser Cys Gln Ala Phe Leu Ala
                1635                1640

Asn Leu Asp Glu Phe Ala Glu Asp Ile Phe Leu Asn Gly Cys
1645                1650                1655
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 81,

Please delete lines 18-47, and insert therefor:

```
ATG GCC GAC ATC CAG ATG ACT CAG TCT CCA                    30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                  10

GCT TCA CTG TCT GCA TCT GTG GGA GAA ACT                    60
Ala Ser Leu Ser Ala Ser Val Gly Glu Thr
            15                  20

GTC ACC ATC ACA TGT GGA GCA AGT GAG AAT                    90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
            25                  30

ATT TAC GGT GCT TTA AAT TGG TAT CAG CGG                   120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Arg
            35                  40

AAA CAG GGA AAA TCT CCT CAG CTC CTG ATC                   150
Lys Gln Gly Lys Ser Pro Gln Leu Leu Ile
            45                  50

TAT GGT GCA ACC AAC TTG GCA GAT GGC ATG                   180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Met
            55                  60

TCA TCG AGG TTC AGT GGC AGT GGA TCT GGT                   210
Ser Ser Arg Phe Ser Gly Ser Gly Ser Gly
            65                  70

AGA CAG TAT TAT CTC AAG ATC AGT AGC CTG                   240
Arg Gln Tyr Tyr Leu Lys Ile Ser Ser Leu
            75                  80

CAT CCT GAC GAT GTT GCA ACG TAT TAC TGT                   270
His Pro Asp Asp Val Ala Thr Tyr Tyr Cys
            85                  90

CAA AAT GTG TTA AAT ACT CCT CTC ACG TTC                   300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
            95                 100
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 81,
Please delete line 48, through column 83, line 21, and insert therefor:

```
GGT GCT GGG ACC AAG TTG GAG CTG AAA CGG                    330
Gly Ala Gly Thr Lys Leu Glu Leu Lys Arg
                105                 110

ACC GGA GGT GGC GGG TCG GGT GGC GGG GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120

TCG GGT GGC GGA GGG TCG CAG GTT CAG CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

CAG CAG TCT GGA GCC GAG CTG ATG AAG CCT                    420
Gln Gln Ser Gly Ala Glu Leu Met Lys Pro
                135                 140

GGG GCC TCA GTG AAG ATG TCC TGC AAG GCT                    450
Gly Ala Ser Val Lys Met Ser Cys Lys Ala
                145                 150

ACT GGC TAC ATA TTC AGT AAC TAC TGG ATA                    480
Thr Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAG TGG ATA AAG CAG AGG CCT GGA CAT GGC                    510
Gln Trp Ile Lys Gln Arg Pro Gly His Gly
                165                 170

CTT GAG TGG ATT GGT GAG ATT TTA CCT GGA                    540
Leu Glu Trp Ile Gly Glu Ile Leu Pro Gly
                175                 180

AGT GGT TCT ACT GAG TAC ACT GAG AAC TTC                    570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
                185                 190

AAG GAC AAG GCC GCA TTC ACT GCA GAT ACA                    600
Lys Asp Lys Ala Ala Phe Thr Ala Asp Thr
                195                 200
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 83,

Please delete lines 22-36, and insert therefor:

```
TCC TCC AAC ACA GCC TAC ATG CAA CTC AGC                    630
Ser Ser Asn Thr Ala Tyr Met Gln Leu Ser
                205                 210

AGC CTG ACA TCA GAG GAC TCT GCC GTC TAT                    660
Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr
                215                 220

TAC TGT GCA AGA TAT TTC TTC GGT AGT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCC AAC TGG TAC TTC GAT GTC TGG GGC GCA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Ala
                235                 240

GGG ACC ACG GTC ACC GTC TCC TCA TGA                        747
Gly Thr Thr Val Thr Val Ser Ser
                245
```

Please delete lines 46-57, and insert therefor:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                     30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
  1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                     60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
                15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                     90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
                25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CGT                    120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Arg
                35                  40
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 85,

Please delete lines 1-30, and insert therefor:

```
AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                    150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                 45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                    180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
                 55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                    210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                 65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                    240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                 75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                    270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
                 85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                    300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                 95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                    330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
                105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
                135                 140
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 85,
Please delete lines 31-60, and insert therefor:

```
GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                    570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
                185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
                195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240
```

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 87,

Please delete lines 1-3, and insert therefor:

```
GGA ACC CTG GTC ACT GTC TCG AGC TGA                              747
Gly Thr Leu Val Thr Val Ser Ser
                245
```

Please delete lines 13-36, and insert therefor:

```
ATG GGA ATC CAA GGA GGG TCT GTC CTG TTC                           30
Met Gly Ile Gln Gly Gly Ser Val Leu Phe
-25                 -20

GGG CTG CTG CTC GTC CTG GCT GTC TTC TGC                           60
Gly Leu Leu Leu Val Leu Ala Val Phe Cys
-15                 -10

CAT TCA GGT CAT AGC CTG CAG GAC ATC CAG                           90
His Ser Gly His Ser Leu Gln Asp Ile Gln
-5                   1               5

ATG ACT CAG TCT CCA GCT TCA CTG TCT GCA                          120
Met Thr Gln Ser Pro Ala Ser Leu Ser Ala
                10                  15

TCT GTG GGA GAA ACT GTC ACC ATC ACA TGT                          150
Ser Val Gly Glu Thr Val Thr Ile Thr Cys
                20                  25

GGA GCA AGT GAG AAT ATT TAC GGT GCT TTA                          180
Gly Ala Ser Glu Asn Ile Tyr Gly Ala Leu
                30                  35

AAT TGG TAT CAG CGG AAA CAG GGA AAA TCT                          210
Asn Trp Tyr Gln Arg Lys Gln Gly Lys Ser
                40                  45

CCT CAG CTC CTG ATC TAT GGT GCA ACC AAC                          240
Pro Gln Leu Leu Ile Tyr Gly Ala Thr Asn
                50                  55
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 87,
Please delete line 37, through column 89, line 9, and insert therefor:

```
TTG GCA GAT GGC ATG TCA TCG AGG TTC AGT                    270
Leu Ala Asp Gly Met Ser Ser Arg Phe Ser
                  60                  65

GGC AGT GGA TCT GGT AGA CAG TAT TAT CTC                    300
Gly Ser Gly Ser Gly Arg Gln Tyr Tyr Leu
                  70                  75

AAG ATC AGT AGC CTG CAT CCT GAC GAT GTT                    330
Lys Ile Ser Ser Leu His Pro Asp Asp Val
                  80                  85

GCA ACG TAT TAC TGT CAA AAT GTG TTA AAT                    360
Ala Thr Tyr Tyr Cys Gln Asn Val Leu Asn
                  90                  95

ACT CCT CTC ACG TTC GGT GCT GGG ACC AAG                    390
Thr Pro Leu Thr Phe Gly Ala Gly Thr Lys
                 100                 105

TTG GAG CTG AAA CGA ACT GTG GCT GCA CCA                    420
Leu Glu Leu Lys Arg Thr Val Ala Ala Pro
                 110                 115

TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG                    450
Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
                 120                 125

CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG                    480
Gln Leu Lys Ser Gly Thr Ala Ser Val Val
                 130                 135

TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG                    510
Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu
                 140                 145

GCC AAA GTA CAG TGG AAG GTG GAT AAC GCC                    540
Ala Lys Val Gln Trp Lys Val Asp Asn Ala
                 150                 155
```

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 89,

Please delete lines 10-29, and insert therefor:

```
CTC CAA TCG GGT AAC TCC CAG GAG AGT GTC                    570
Leu Gln Ser Gly Asn Ser Gln Glu Ser Val
                160                 165

ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC                    600
Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
                170                 175

AGC CTC AGC AGC ACC CTG ACG CTG AGC AAA                    630
Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys
                180                 185

GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC                    660
Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
                190                 195

TGC GAA GTC ACC CAT CAG GGC CTG AGC TCG                    690
Cys Glu Val Thr His Gln Gly Leu Ser Ser
                200                 205

CCC GTC ACA AAG AGC TTC AAC AGG GGA GAG                    720
Pro Val Thr Lys Ser Phe Asn Arg Gly Glu
                210                 215

TGT TAG                                                    726
Cys
```

Please delete lines 39-44, and insert therefor:

```
ATG AAA TGG AGC TGG GTT ATT CTC TTC CTC                     30
Met Lys Trp Ser Trp Val Ile Leu Phe Leu
               -15                 -10

CTG TCA GTA ACT GCA GGT GTC CAC TCC CAG                     60
Leu Ser Val Thr Ala Gly Val His Ser Gln
                -5                   1
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 89,
Please delete line 45, through column 91, line 15, and insert therefor:

```
GTT CAG CTG CAG CAG TCT GGA GCT GAG CTG                    90
Val Gln Leu Gln Gln Ser Gly Ala Glu Leu
                5                   10

ATG AAG CCT GGG GCC TCA GTG AAG ATG TCC                   120
Met Lys Pro Gly Ala Ser Val Lys Met Ser
            15                  20

TGC AAG GCT ACT GGC TAC ATA TTC AGT AAC                   150
Cys Lys Ala Thr Gly Tyr Ile Phe Ser Asn
            25                  30

TAC TGG ATA CAG TGG ATA AAG CAG AGG CCT                   180
Tyr Trp Ile Gln Trp Ile Lys Gln Arg Pro
            35                  40

GGA CAT GGC CTT GAG TGG ATT GGT GAG ATT                   210
Gly His Gly Leu Glu Trp Ile Gly Glu Ile
            45                  50

TTA CCT GGA AGT GGT TCT ACT GAG TAC ACT                   240
Leu Pro Gly Ser Gly Ser Thr Glu Tyr Thr
            55                  60

GAG AAC TTC AAG GAC AAG GCC GCA TTC ACT                   270
Glu Asn Phe Lys Asp Lys Ala Ala Phe Thr
            65                  70

GCA GAT ACA TCC TCC AAC ACA GCC TAC ATG                   300
Ala Asp Thr Ser Ser Asn Thr Ala Tyr Met
            75                  80

CAA CTC AGC AGC CTG ACA TCA GAG GAC TCT                   330
Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser
            85                  90

GCC GTC TAT TAC TGT GCA AGA TAT TTC TTC                   360
Ala Val Tyr Tyr Cys Ala Arg Tyr Phe Phe
            95                  100
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 91,
Please delete lines 16-45, and insert therefor:

```
GGT AGT AGC CCC AAC TGG TAC TTC GAT GTC                    390
Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
            105                 110

TGG GGC GCA GGG ACC ACG GTC ACC GTC TCC                    420
Trp Gly Ala Gly Thr Thr Val Thr Val Ser
            115                 120

TCA GCC TCC ACC AAG GGC CCA TCG GTC TTC                    450
Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
            125                 130

CCC CTG GCG CCC TCC TCC AAG AGC ACC TCT                    480
Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
            135                 140

GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC                    510
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
            145                 150

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG                    540
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
            155                 160

TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC                    570
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
            165                 170

GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC                    600
Val His Thr Phe Pro Ala Val Leu Gln Ser
            175                 180

TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG                    630
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
            185                 190

ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG                    660
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
            195                 200
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 91,
Please delete lines 46-54, and insert therefor:

```
ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC                    690
Thr Tyr Ile Cys Asn Val Asn His Lys Pro
            205                 210

AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG                    720
Ser Asn Thr Lys Val Asp Lys Lys Val Glu
            215                 220

CCC AAA TCT TGT GAC AAA ACT CAC ACA TAA                    750
Pro Lys Ser Cys Asp Lys Thr His Thr
            225
```

Column 93
Please delete lines 4-21, and insert therefor:

```
ATG AAG TGG AGC TGG GTT ATT CTC TTC CTC                     30
Met Lys Trp Ser Trp Val Ile Leu Phe Leu
               -15                 -10

CTG TCA GTA ACT GCC GGC GTC CAC TCC CAA                     60
Leu Ser Val Thr Ala Gly Val His Ser Gln
                -5                   1

GTC CAA CTG GTG CAA TCC GGC GCC GAG GTC                     90
Val Gln Leu Val Gln Ser Gly Ala Glu Val
             5                  10

AAG AAG CCA GGG GCC TCA GTC AAA GTG TCC                    120
Lys Lys Pro Gly Ala Ser Val Lys Val Ser
            15                  20

TGT AAA GCT AGC GGC TAT ATT TTT TCT AAT                    150
Cys Lys Ala Ser Gly Tyr Ile Phe Ser Asn
            25                  30

TAT TGG ATT CAA TGG GTG CGT CAG GCC CCC                    180
Tyr Trp Ile Gln Trp Val Arg Gln Ala Pro
            35                  40
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 93,
Please delete lines 22-51, and insert therefor:

```
GGG CAG GGC CTG GAA TGG ATG GGT GAG ATC                    210
Gly Gln Gly Leu Glu Trp Met Gly Glu Ile
             45                  50

TTA CCG GGC TCT GGT AGC ACC GAA TAT GCC                    240
Leu Pro Gly Ser Gly Ser Thr Glu Tyr Ala
             55                  60

CAA AAA TTC CAG GGC CGT GTT ACT ATG ACT                    270
Gln Lys Phe Gln Gly Arg Val Thr Met Thr
             65                  70

GCG GAC ACT TCG ACT AGT ACA GCC TAC ATG                    300
Ala Asp Thr Ser Thr Ser Thr Ala Tyr Met
             75                  80

GAG CTC TCC AGC CTG CGA TCG GAG GAC ACG                    330
Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr
             85                  90

GCC GTC TAT TAT TGC GCG CGT TAT TTT TTT                    360
Ala Val Tyr Tyr Cys Ala Arg Tyr Phe Phe
             95                 100

GGT TCT AGC CCG AAT TGG TAT TTT GAT GTT                    390
Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
            105                 110

TGG GGT CAA GGA ACC CTG GTC ACT GTC TCG                    420
Trp Gly Gln Gly Thr Leu Val Thr Val Ser
            115                 120

AGC GCC TCC ACC AAG GGC CCA TCG GTC TTC                    450
Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
            125                 130

CCC CTG GCG CCC TCC TCC AAG AGC ACC TCT                    480
Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
            135                 140
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 93,

Please delete line 52, through column 95, line 27, and insert therefor:

```
GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC                    510
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
            145                 150

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG                    540
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
            155                 160

TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC                    570
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
            165                 170

GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC                    600
Val His Thr Phe Pro Ala Val Leu Gln Ser
            175                 180

TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG                    630
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
            185                 190

ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG                    660
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
            195                 200

ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC                    690
Thr Tyr Ile Cys Asn Val Asn His Lys Pro
            205                 210

AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG                    720
Ser Asn Thr Lys Val Asp Lys Lys Val Glu
            215                 220

CCC AAA TCT TGT GAC AAA ACT CAC ACA TAA                    750
Pro Lys Ser Cys Asp Lys Thr His Thr
            225                 230
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 95,

Please delete line 31, through column 97, line 3, and insert therefor:

```
ATG AAG TGG AGC TGG GTT ATT CTC TTC CTC                    30
Met Lys Trp Ser Trp Val Ile Leu Phe Leu
               -15                 -10

CTG TCA GTA ACT GCC GGC GTC CAC TCC CAA                    60
Leu Ser Val Thr Ala Gly Val His Ser Gln
                -5                   1

GTC CAA CTG GTG CAA TCC GGC GCC GAG GTC                    90
Val Gln Leu Val Gln Ser Gly Ala Glu Val
             5                   10

AAG AAG CCA GGG GCC TCA GTC AAA GTG TCC                   120
Lys Lys Pro Gly Ala Ser Val Lys Val Ser
            15                  20

TGT AAA GCT AGC GGC TAT ATT TTT TCT AAT                   150
Cys Lys Ala Ser Gly Tyr Ile Phe Ser Asn
            25                  30

TAT TGG ATT CAA TGG GTG CGT CAG GCC CCC                   180
Tyr Trp Ile Gln Trp Val Arg Gln Ala Pro
            35                  40

GGG CAG GGC CTG GAA TGG ATG GGT GAG ATC                   210
Gly Gln Gly Leu Glu Trp Met Gly Glu Ile
            45                  50

TTA CCG GGC TCT GGT AGC ACC GAA TAT ACC                   240
Leu Pro Gly Ser Gly Ser Thr Glu Tyr Thr
            55                  60

GAA AAT TTT AAA GAC CGT GTT ACT ATG ACG                   270
Glu Asn Phe Lys Asp Arg Val Thr Met Thr
            65                  70

CGT GAC ACT TCG ACT AGT ACA GTA TAC ATG                   300
Arg Asp Thr Ser Thr Ser Thr Val Tyr Met
            75                  80
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 97,

Please delete lines 4-33, and insert therefor:

```
GAG CTC TCC AGC CTG CGA TCG GAG GAC ACG                    330
Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr
             85                  90

GCC GTC TAT TAT TGC GCG CGT TAT TTT TTT                    360
Ala Val Tyr Tyr Cys Ala Arg Tyr Phe Phe
             95                 100

GGT TCT AGC CCG AAT TGG TAT TTT GAT GTT                    390
Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
            105                 110

TGG GGT CAA GGA ACC CTG GTC ACT GTC TCG                    420
Trp Gly Gln Gly Thr Leu Val Thr Val Ser
            115                 120

AGC GCC TCC ACC AAG GGC CCA TCG GTC TTC                    450
Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
            125                 130

CCC CTG GCG CCC TCC TCC AAG AGC ACC TCT                    480
Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
            135                 140

GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC                    510
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
            145                 150

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG                    540
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
            155                 160

TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC                    570
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
            165                 170

GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC                    600
Val His Thr Phe Pro Ala Val Leu Gln Ser
            175                 180
```

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 97,

Please delete lines 34-48, and insert therefor:

```
TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG                    630
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
            185                 190

ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG                    660
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
            195                 200

ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC                    690
Thr Tyr Ile Cys Asn Val Asn His Lys Pro
            205                 210

AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG                    720
Ser Asn Thr Lys Val Asp Lys Lys Val Glu
            215                 220

CCC AAA TCT TGT GAC AAA ACT CAC ACA TAA                    750
Pro Lys Ser Cys Asp Lys Thr His Thr
            225                 230
```

Column 99,

Please delete lines 2-13, and insert therefor:

```
ATG GGA ATC CAA GGA GGG TCT GTC CTG TTC                     30
Met Gly Ile Gln Gly Gly Ser Val Leu Phe
-25                 -20

GGG CTG CTG CTC GTC CTG GCT GTC TTC TGC                     60
Gly Leu Leu Leu Val Leu Ala Val Phe Cys
-15                 -10

CAT TCA GGT CAT AGC CTG CAG GAT ATC CAG                     90
His Ser Gly His Ser Leu Gln Asp Ile Gln
-5                   1               5

ATG ACC CAG TCC CCG TCC TCC CTG TCC GCC                    120
Met Thr Gln Ser Pro Ser Ser Leu Ser Ala
                10                  15
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 99,
Please delete lines 14-43, and insert therefor:

```
TCT GTG GGC GAT AGG GTC ACC ATC ACC TGC                    150
Ser Val Gly Asp Arg Val Thr Ile Thr Cys
                  20                  25

GGC GCC AGC GAA AAC ATC TAT GGC GCG CTG                    180
Gly Ala Ser Glu Asn Ile Tyr Gly Ala Leu
                  30                  35

AAC TGG TAT CAA CGT AAA CCT GGG AAA GCT                    210
Asn Trp Tyr Gln Arg Lys Pro Gly Lys Ala
                  40                  45

CCG AAG CTT CTG ATT TAC GGT GCG ACG AAC                    240
Pro Lys Leu Leu Ile Tyr Gly Ala Thr Asn
                  50                  55

CTG GCA GAT GGA GTC CCT TCT CGC TTC TCT                    270
Leu Ala Asp Gly Val Pro Ser Arg Phe Ser
                  60                  65

GGA TCC GGC TCC GGA ACG GAT TAC ACT CTG                    300
Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu
                  70                  75

ACC ATC AGC AGT CTG CAA CCT GAG GAC TTC                    330
Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe
                  80                  85

GCT ACG TAT TAC TGT CAG AAC GTT TTA AAT                    360
Ala Thr Tyr Tyr Cys Gln Asn Val Leu Asn
                  90                  95

ACT CCG TTG ACT TTC GGA CAG GGT ACC AAG                    390
Thr Pro Leu Thr Phe Gly Gln Gly Thr Lys
                 100                 105

GTG GAA ATA AAA CGA ACT GTG GCT GCA CCA                    420
Val Glu Ile Lys Arg Thr Val Ala Ala Pro
                 110                 115
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 99,

Please delete line 44, through column 101, line 17, and insert therefor:

```
TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG                    450
Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
                120                 125

CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG                    480
Gln Leu Lys Ser Gly Thr Ala Ser Val Val
                130                 135

TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG                    510
Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu
                140                 145

GCC AAA GTA CAG TGG AAG GTG GAT AAC GCC                    540
Ala Lys Val Gln Trp Lys Val Asp Asn Ala
                150                 155

CTC CAA TCG GGT AAC TCC CAG GAG AGT GTC                    570
Leu Gln Ser Gly Asn Ser Gln Glu Ser Val
                160                 165

ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC                    600
Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
                170                 175

AGC CTC AGC AGC ACC CTG ACG CTG AGC AAA                    630
Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys
                180                 185

GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC                    660
Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
                190                 195

TGC GAA GTC ACC CAT CAG GGC CTG AGC TCG                    690
Cys Glu Val Thr His Gln Gly Leu Ser Ser
                200                 205

CCC GTC ACA AAG AGC TTC AAC AGG GGA GAG                    720
Pro Val Thr Lys Ser Phe Asn Arg Gly Glu
                210                  215

TGT TAG                                                    726
Cys
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 101,
Please delete lines 28-57, and insert therefor:

```
ATG GGA ATC CAA GGA GGG TCT GTC CTG TTC                    30
Met Gly Ile Gln Gly Gly Ser Val Leu Phe
-25                 -20

GGG CTG CTG CTC GTC CTG GCT GTC TTC TGC                    60
Gly Leu Leu Leu Val Leu Ala Val Phe Cys
-15                 -10

CAT TCA GGT CAT AGC CTG CAG GAT ATC CAG                    90
His Ser Gly His Ser Leu Gln Asp Ile Gln
-5                   1               5

ATG ACC CAG TCC CCG TCC TCC CTG TCC GCC                   120
Met Thr Gln Ser Pro Ser Ser Leu Ser Ala
                10                  15

TCT GTG GGC GAT AGG GTC ACC ATC ACC TGC                   150
Ser Val Gly Asp Arg Val Thr Ile Thr Cys
                20                  25

GGC GCC AGC GAA AAC ATC TAT GGC GCG CTG                   180
Gly Ala Ser Glu Asn Ile Tyr Gly Ala Leu
                30                  35

AAC TGG TAT CAA CGT AAA CCT GGG AAA GCT                   210
Asn Trp Tyr Gln Arg Lys Pro Gly Lys Ala
                40                  45

CCG AAG CTT CTG ATT TAC GGT GCG ACG AAC                   240
Pro Lys Leu Leu Ile Tyr Gly Ala Thr Asn
                50                  55

CTG GCA GAT GGA GTC CCT TCT CGC TTC TCT                   270
Leu Ala Asp Gly Val Pro Ser Arg Phe Ser
                60                  65

GGA TCC GGC TCC GGA ACG GAT TTC ACT CTG                   300
Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu
                70                  75
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 103,

Please delete lines 1-30, and insert therefor:

```
ACC ATC AGC AGT CTG CAG CCT GAA GAC TTC                    330
Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe
                 80                  85

GCT ACG TAT TAC TGT CAG AAC GTT TTA AAT                    360
Ala Thr Tyr Tyr Cys Gln Asn Val Leu Asn
                 90                  95

ACT CCG TTG ACT TTC GGA CAG GGT ACC AAG                    390
Thr Pro Leu Thr Phe Gly Gln Gly Thr Lys
                100                 105

GTG GAA ATA AAA CGA ACT GTG GCT GCA CCA                    420
Val Glu Ile Lys Arg Thr Val Ala Ala Pro
                110                 115

TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG                    450
Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
                120                 125

CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG                    480
Gln Leu Lys Ser Gly Thr Ala Ser Val Val
                130                 135

TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG                    510
Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu
                140                 145

GCC AAA GTA CAG TGG AAG GTG GAT AAC GCC                    540
Ala Lys Val Gln Trp Lys Val Asp Asn Ala
                150                 155

CTC CAA TCG GGT AAC TCC CAG GAG AGT GTC                    570
Leu Gln Ser Gly Asn Ser Gln Glu Ser Val
                160                 165

ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC                    600
Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
                170                 175
```

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 103,

Please delete lines 31-44, and insert therefor:

```
AGC CTC AGC AGC ACC CTG ACG CTG AGC AAA                    630
Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys
                180                 185

GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC                    660
Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
                190                 195

TGC GAA GTC ACC CAT CAG GGC CTG AGC TCG                    690
Cys Glu Val Thr His Gln Gly Leu Ser Ser
                200                 205

CCC GTC ACA AAG AGC TTC AAC AGG GGA GAG                    720
Pro Val Thr Lys Ser Phe Asn Arg Gly Glu
                210                 215

TGT TAG                                                    726
Cys
```

Please delete line 55, through column 105, line 9, and insert therefor:

```
ATG GAC ATG AGG GTC CCC GCT CAG CTC CTG                     30
Met Asp Met Arg Val Pro Ala Gln Leu Leu
            -20                 -15

GGG CTC CTG CTA CTC TGG CTC CGA GGT GCC                     60
Gly Leu Leu Leu Leu Trp Leu Arg Gly Ala
            -10                  -5

AGA TGT GAT ATC CAG ATG ACC CAG TCC CCG                     90
Arg Cys Asp Ile Gln Met Thr Gln Ser Pro
          1                5

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                    120
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
     10                  15
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 105,

Please delete lines 10-39, and insert therefor:

```
GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                    150
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
     20                  25

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                    180
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
     30                  35

AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                    210
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
     40                  45

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                    240
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
     50                  55

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                    270
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
     60                  65

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                    300
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
     70                  75

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                    330
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
     80                  85

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                    360
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
     90                  95

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGA                    390
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
    100                 105

ACT GTG GCT GCA CCA TCT GTC TTC ATC TTC                    420
Thr Val Ala Ala Pro Ser Val Phe Ile Phe
    110                 115
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 105,
Please delete line 40, through column 107, line 9, and insert therefor:

```
CCG CCA TCT GAT GAG CAG TTG AAA TCT GGA                    450
Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
    120                 125

ACT GCC TCT GTT GTG TGC CTG CTG AAT AAC                    480
Thr Ala Ser Val Val Cys Leu Leu Asn Asn
    130                 135

TTC TAT CCC AGA GAG GCC AAA GTA CAG TGG                    510
Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp
    140                 145

AAG GTG GAT AAC GCC CTC CAA TCG GGT AAC                    540
Lys Val Asp Asn Ala Leu Gln Ser Gly Asn
    150                 155

TCC CAG GAG AGT GTC ACA GAG CAG GAC AGC                    570
Ser Gln Glu Ser Val Thr Glu Gln Asp Ser
    160                 165

AAG GAC AGC ACC TAC AGC CTC AGC AGC ACC                    600
Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr
    170                 175

CTG ACG CTG AGC AAA GCA GAC TAC GAG AAA                    630
Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys
    180                 185

CAC AAA GTC TAC GCC TGC GAA GTC ACC CAT                    660
His Lys Val Tyr Ala Cys Glu Val Thr His
    190                  195

CAG GGC CTG AGC TCG CCC GTC ACA AAG AGC                    690
Gln Gly Leu Ser Ser Pro Val Thr Lys Ser
    200                 205

TTC AAC AGG GGA GAG TGT TAG                                711
Phe Asn Arg Gly Glu Cys
    210
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 107,
Please delete lines 20-49, and insert therefor:

```
ATG AAG TGG AGC TGG GTT ATT CTC TTC CTC                    30
Met Lys Trp Ser Trp Val Ile Leu Phe Leu
               -15                 -10

CTG TCA GTA ACT GCC GGC GTC CAC TCC CAA                    60
Leu Ser Val Thr Ala Gly Val His Ser Gln
                -5                   1

GTC CAA CTG GTG CAA TCC GGC GCC GAG GTC                    90
Val Gln Leu Val Gln Ser Gly Ala Glu Val
                5                   10

AAG AAG CCA GGG GCC TCA GTC AAA GTG TCC                   120
Lys Lys Pro Gly Ala Ser Val Lys Val Ser
               15                   20

TGT AAA GCT AGC GGC TAT ATT TTT TCT AAT                   150
Cys Lys Ala Ser Gly Tyr Ile Phe Ser Asn
               25                   30

TAT TGG ATT CAA TGG GTG CGT CAG GCC CCC                   180
Tyr Trp Ile Gln Trp Val Arg Gln Ala Pro
               35                   40

GGG CAG GGC CTG GAA TGG ATG GGT GAG ATC                   210
Gly Gln Gly Leu Glu Trp Met Gly Glu Ile
               45                   50

TTA CCG GGC TCT GGT AGC ACC GAA TAT GCC                   240
Leu Pro Gly Ser Gly Ser Thr Glu Tyr Ala
               55                   60

CAA AAA TTC CAG GGC CGT GTT ACT ATG ACT                   270
Gln Lys Phe Gln Gly Arg Val Thr Met Thr
               65                   70

CGT GAC ACT TCG ACT AGT ACA GTA TAC ATG                   300
Arg Asp Thr Ser Thr Ser Thr Val Tyr Met
               75                   80
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 107,
Please delete line 50, through column 109, line 21, and insert therefor:

```
GAG CTC TCC AGC CTG CGA TCG GAG GAC ACG                    330
Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr
             85                  90

GCC GTC TAT TAT TGC GCG CGT TAT TTT TTT                    360
Ala Val Tyr Tyr Cys Ala Arg Tyr Phe Phe
             95                 100

GGT TCT AGC CCG AAT TGG TAT TTT GAT GTT                    390
Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
            105                 110

TGG GGT CAA GGA ACC CTG GTC ACT GTC TCG                    420
Trp Gly Gln Gly Thr Leu Val Thr Val Ser
            115                 120

AGC GCC TCC ACC AAG GGC CCA TCG GTC TTC                    450
Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
            125                 130

CCC CTG GCG CCC TCC TCC AAG AGC ACC TCT                    480
Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
            135                 140

GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC                    510
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
            145                 150

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG                    540
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
            155                 160

TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC                    570
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
            165                 170

GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC                    600
Val His Thr Phe Pro Ala Val Leu Gln Ser
            175                 180
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 109,

Please delete lines 22-36, and insert therefor:

```
TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG                    630
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
            185                 190

ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG                    660
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
            195                 200

ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC                    690
Thr Tyr Ile Cys Asn Val Asn His Lys Pro
            205                 210

AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG                    720
Ser Asn Thr Lys Val Asp Lys Lys Val Glu
            215                 220

CCC AAA TCT TGT GAC AAA ACT CAC ACA TAA                    750
Pro Lys Ser Cys Asp Lys Thr His Thr
            225                 230
```

Please delete lines 47-58, and insert therefor:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                     30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
  1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                     60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
                15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                     90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
                25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                    120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
                35                  40
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 111,

Please delete lines 1-30, and insert therefor:

```
AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                    150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                 45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                    180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
                 55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                    210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                 65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                    240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                 75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                    270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
                 85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                    300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                 95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                    330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
                105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
                135                 140
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 111,
Please delete lines 31-60, and insert therefor:

```
GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180

TCT GGT AGC ACC GAA TAT GCC CAA AAA TTC                    570
Ser Gly Ser Thr Glu Tyr Ala Gln Lys Phe
                185                 190

CAG GGC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Gln Gly Arg Val Thr Met Thr Arg Asp Thr
                195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240
```

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 113,
Please delete lines 1-3, and insert therefor:

```
GGA ACC CTG GTC ACT GTC TCG AGC TGA                              747
Gly Thr Leu Val Thr Val Ser Ser
                245
```

Column 119,
Please delete lines 2-25, and insert therefor:

```
ATG GCC AAT ATT GTG CTG ACC CAA TCT CCA                           30
Met Ala Asn Ile Val Leu Thr Gln Ser Pro
  1               5                  10

GCT TCT TTG GCT GTG TCT CTA GGG CAG AGG                           60
Ala Ser Leu Ala Val Ser Leu Gly Gln Arg
                15                  20

GCC ACC ATA TCC TGC AGA GCC AGT GAA AGT                           90
Ala Thr Ile Ser Cys Arg Ala Ser Glu Ser
                25                  30

GTT GAT AGT TAT GAC AAT AGT TTT ATG CAC                          120
Val Asp Ser Tyr Asp Asn Ser Phe Met His
                35                  40

TGG TAC CAG CAG AAA CCA GGA CAG CCA CCC                          150
Trp Tyr Gln Gln Lys Pro Gly Gln Pro Pro
                45                  50

AAA CTC CTC ATC TTT CTT GCA TCC AAC CTA                          180
Lys Leu Leu Ile Phe Leu Ala Ser Asn Leu
                55                  60

GAA TCT GGG GTC CCT GCC AGG TTC AGT GGC                          210
Glu Ser Gly Val Pro Ala Arg Phe Ser Gly
                65                  70

AGT GGG TCT AGG ACA GAC TTC ACC CTC ACC                          240
Ser Gly Ser Arg Thr Asp Phe Thr Leu Thr
                75                  80
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 119,
Please delete lines 26-55, and insert therefor:

```
ATT GAT CCT GTG GAG GCT GAT GAT GCT GCA                    270
Ile Asp Pro Val Glu Ala Asp Asp Ala Ala
                 85                  90

ACC TAT TAC TGT CAG CAA AAT AAT GAG GTT                    300
Thr Tyr Tyr Cys Gln Gln Asn Asn Glu Val
                 95                 100

CCG AAC ACG TTC GGA GGG GGG ACC AAG CTG                    330
Pro Asn Thr Phe Gly Gly Gly Thr Lys Leu
                105                 110

GAA ATA AAA CGG ACC GGA GGT GGC GGG TCG                    360
Glu Ile Lys Arg Thr Gly Gly Gly Gly Ser
                115                 120

GGT GGC GGG GGA TCG GGT GGC GGA GGG TCG                    390
Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
                125                 130

GAC GTC AAG CTC GTG GAG TCT GGG GGA GAC                    420
Asp Val Lys Leu Val Glu Ser Gly Gly Asp
                135                 140

TTA GTG AAG CTT GGA GGG TCC CTG AAA CTC                    450
Leu Val Lys Leu Gly Gly Ser Leu Lys Leu
                145                 150

TCC TGT GCA GCC TCT GGA TTC ACC TTC AGT                    480
Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
                155                 160

AGC TAT TAT ATG TCT TGG GTT CGC CAG ATT                    510
Ser Tyr Tyr Met Ser Trp Val Arg Gln Ile
                165                 170

TCA GAG AAG AGG CTG GAG TTG GTC GCA GCC                    540
Ser Glu Lys Arg Leu Glu Leu Val Ala Ala
                175                 180
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 119,
Please delete lines 56, through column 121, line 22, and insert therefor:

```
ATT AAT AGT AAT GGT GAT AGC ACC TAC TAT                    570
Ile Asn Ser Asn Gly Asp Ser Thr Tyr Tyr
                185                 190

CCA GAC ACT GTG AAG GGC CGA TTC ACC ATC                    600
Pro Asp Thr Val Lys Gly Arg Phe Thr Ile
                195                 200

TCC AGA GAC AAT GCC AAG AGC ACC CTG GAT                    630
Ser Arg Asp Asn Ala Lys Ser Thr Leu Asp
                205                 210

CTG CAA ATG AGC AGT CTG AAG TCT GAG GAC                    660
Leu Gln Met Ser Ser Leu Lys Ser Glu Asp
                215                 220

ACA GCC TTG TAT TTC TGT GTA AGA GAG ACT                    690
Thr Ala Leu Tyr Phe Cys Val Arg Glu Thr
                225                 230

TAT TAC TAC GGG ATT AGT CCC GTC TTC GAT                    720
Tyr Tyr Tyr Gly Ile Ser Pro Val Phe Asp
                235                 240

GTC TGG GGC ACA GGG ACC ACG GTC ACC GTC                    750
Val Trp Gly Thr Gly Thr Thr Val Thr Val
                245                 250

TCC TCA CTC GAG CAC CAC CAC CAC CAC CAC                    780
Ser Ser Leu Glu His His His His His His
                255                 260

TGA                                                        783
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 121,
Please delete lines 32, through column 123, line 3, and insert therefor:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                    30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
  1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                    60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
                15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                    90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
                25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                   120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
                35                  40

AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                   150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                   180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
                55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                   210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                   240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                   270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
                85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                   300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                95                 100
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 123,
Please delete lines 4-33, and insert therefor:

```
GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                    330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
                105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
                135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                    570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
                185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
                195                 200
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 123,
Please delete lines 34-48, and insert therefor:

```
TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                        747
Gly Thr Leu Val Thr Val Ser Ser
                245
```

Column 125,
Please delete lines 2-13, and insert therefor:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                     30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
  1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                     60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
                15                  20

GTC ACC ATC ACC TGC CGT GCT AGC GAA AAC                     90
Val Thr Ile Thr Cys Arg Ala Ser Glu Asn
                25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                    120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
                35                  40
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 125,
Please delete lines 14-43, and insert therefor:

```
AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                    150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                 45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                    180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
                 55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                    210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                 65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                    240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                 75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                    270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
                 85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                    300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                 95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                    330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
                105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
                135                 140
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 125,
Please delete line 44, through column 127, line 15, and insert therefor:

```
GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180

TCT GGT AGC ACC GAA TAT GCC CAA AAA TTC                    570
Ser Gly Ser Thr Glu Tyr Ala Gln Lys Phe
                185                 190

CAG GGC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Gln Gly Arg Val Thr Met Thr Arg Asp Thr
                195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240
```

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 127,

Please delete lines 16-18, and insert therefor:

```
GGA ACC CTG GTC ACT GTC TCG AGC TGA                              747
Gly Thr Leu Val Thr Val Ser Ser
                245
```

Please delete lines 29-52, and insert therefor:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                           30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
  1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                           60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
                15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                           90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
                25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                          120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
                35                  40

AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                          150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                45                  50

TAC GGT GCG ACG AGC CTG CAG TCT GGA GTC                          180
Tyr Gly Ala Thr Ser Leu Gln Ser Gly Val
                55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                          210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                          240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                75                  80
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 127,
Please delete line 53, through column 129, line 24, and insert therefor:

```
CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                    270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
                 85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                    300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                 95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                    330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
                105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
                135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 129,

Please delete lines 25-45, and insert therefor:

```
TCT GGT AGC ACC GAA TAT GCC CAA AAA TTC                570
Ser Gly Ser Thr Glu Tyr Ala Gln Lys Phe
                185                 190

CAG GGC CGT GTT ACT ATG ACG CGT GAC ACT                600
Gln Gly Arg Val Thr Met Thr Arg Asp Thr
                195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                    747
Gly Thr Leu Val Thr Val Ser Ser
                245
```

Please delete line 56, through column 131, line 3, and insert therefor:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                 30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
  1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                 60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
                15                  20
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 131,
Please delete lines 4-33, and insert therefor:

```
GTC ACC ATC ACC TGC CGT GCT AGC GAA AAC                    90
Val Thr Ile Thr Cys Arg Ala Ser Glu Asn
                 25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                   120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
                 35                  40

AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                   150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                 45                  50

TAC GGT GCG ACG AGC CTG CAG TCT GGA GTC                   180
Tyr Gly Ala Thr Ser Leu Gln Ser Gly Val
                 55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                   210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                 65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                   240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                 75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                   270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
                 85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                   300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                 95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                   330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
                105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                   360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 131,
Please delete line 34, through column 133, line 3, and insert therefor:

```
TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
                135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180

TCT GGT AGC ACC GAA TAT GCC CAA AAA TTC                    570
Ser Gly Ser Thr Glu Tyr Ala Gln Lys Phe
                185                 190

CAG GGC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Gln Gly Arg Val Thr Met Thr Arg Asp Thr
                195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 133,

Please delete lines 4-12, and insert therefor:

```
TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                        747
Gly Thr Leu Val Thr Val Ser Ser
                245
```

Please delete lines 22-45, and insert therefor:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                     30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
  1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                     60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
                15                  20

GTC ACC ATC ACC TGC CGT GCT AGC GAA AAC                     90
Val Thr Ile Thr Cys Arg Ala Ser Glu Asn
                25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                    120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
                35                  40

AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                    150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                    180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
                55                  60
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 133,

Please delete line 46, through column 135, line 12, and insert therefor:

```
CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                    210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                 65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                    240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                 75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                    270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
                 85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                    300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                 95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                    330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
                105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
                135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 135,

Please delete lines 13-39, and insert therefor:

```
CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                    570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
                185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
                195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                        747
Gly Thr Leu Val Thr Val Ser Ser
                245
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 135,
Please delete line 49, through column 137, line 21, and insert therefor:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                    30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
  1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                    60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
                15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                    90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
                25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                   120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
                35                  40

AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                   150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                45                  50

TAC GGT GCG ACG AGC CTG CAG TCT GGA GTC                   180
Tyr Gly Ala Thr Ser Leu Gln Ser Gly Val
                55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                   210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                   240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                   270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
                85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                   300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                95                 100
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 137,
Please delete lines 22-51, and insert therefor:

```
GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                    330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
                105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
                135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                    570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
                185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
                195                 200
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 137,
Please delete line 52, through column 139, line 6, and insert therefor:

```
TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                        747
Gly Thr Leu Val Thr Val Ser Ser
                245
```

Column 139,
Please delete lines 16-27, and insert therefor:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                     30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
  1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                     60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
                15                  20

GTC ACC ATC ACC TGC CGT GCT AGC GAA AAC                     90
Val Thr Ile Thr Cys Arg Ala Ser Glu Asn
                25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                    120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
                35                  40
```

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 139,

Please delete lines 28-57, and insert therefor:

```
AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                    150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                45                  50

TAC GGT GCG ACG AGC CTG CAG TCT GGA GTC                    180
Tyr Gly Ala Thr Ser Leu Gln Ser Gly Val
                55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                    210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                    240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                    270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
                85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                    300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                    330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
               105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
               115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
               125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
               135                 140
```

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 141,
Please delete lines 1-30, and insert therefor:

```
GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                    570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
                185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
                195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240
```

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 141,
Please delete lines 31-33, and insert therefor:

```
GGA ACC CTG GTC ACT GTC TCG AGC TGA                    747
Gly Thr Leu Val Thr Val Ser Ser
                245
```

Signed and Sealed this

Nineteenth Day of November, 2002

Attest:

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

Attesting Officer

AML is approximately 1%. The higher frequency of the R513Q polymorphism in sporadic AML might imply an increased susceptibility to childhood AML in children carrying this variant.

4

Prodaptin-linked CD59, a membrane-targeted recombinant CD59, coats PNH red cells *in vitro* and protects murine red cells from human complement mediated lysis when given *in vivo*

A. Sah,[1] S. H. Ridley,[2] S. J. Richards,[1] M. J. Cullen,[1] R. Jones,[1] R. G. Oldroyd,[2] J. White,[2] D. Esser[2] & P. Hillmen[1]

[1]*HMDS, Leeds Teaching Hospitals NHS Trust, Leeds;* [2]*Adprotech Ltd, Essex, UK*

Paroxysmal nocturnal haemoglobinuria (PNH) is characterized by intravascular haemolysis, venous thrombosis and is associated with aplastic anaemia. PNH cells are deficient in glycosylphosphatidylinositol-linked antigens. The haemolysis and thrombosis result from increased complement sensitivity of PNH cells because they lack complement regulatory molecules, particularly CD59. CD59 deficiency from PNH red cells can be complete (type III) or partial (type II) cells. Patients with only type II cells do not haemolyse, indicating that low levels of CD59 control complement activity. Replacing CD59 on PNH cells should prevent complement-mediated haemolysis and possibly thrombosis. Prodaptin-CD59 is a recombinant human CD59, which has been modified using site-specific attachment of a membrane-interactive acylated, charged peptide instead of the GPI anchor (Prodaptin™ technology). Prodaptin-CD59 is effective in complement lysis assays significantly reducing haemolysis compared with unmodified recombinant sCD59 with comparable activity to native CD59. Whole blood from eight PNH patients was incubated with Prodaptin-CD59, at various concentrations and incubation times. PNH erythrocytes were coated with CD59 to levels similar to type II cells at concentrations of 100 nmol/l Prodaptin-CD59 in all patients. Normal levels of CD59 were achieved at concentrations of 1000 nmol/l. PNH type III cells were completely coated by CD59 after 1 min with persistent CD59 expression after 3 d storage at 4 and 37°C. In order to assess Prodaptin-CD59 *in vivo*, groups of CD1 mice were treated with various concentrations of Prodaptin-CD59 by i.v. injection. Blood was taken 15 min to 24 h after injection and exposed to human complement. The erythrocytes from mice given 10 mg/kg Prodaptin-CD59 were protected from lysis up to the 6-h time point. Erythrocytes from the 50-mg/kg group were protected from lysis up to and including the 24 h time point ($P = 0.002$). No toxicity was observed. Prodaptin-CD59 represents a potential novel therapeutic approach for patients with PNH and merits further investigation.

5

Eculizumab, a C5 complement-blocking antibody, controls haemolysis in paroxysmal nocturnal haemoglobinuria (PNH) with responses maintained over a prolonged period of therapy

P. Hillmen,[1] C. Hall,[1] J. C. W. Marsh,[2] M. Elebute,[2] S. J. Richards,[1] S. A. Rollins,[3] C. F. Mojcik[3] & R. P. Rother[3]

[1]*Department of Haematology, Leeds Teaching Hospitals NHS Trust, Leeds;* [2]*Department of Haematology, St George's Hospital Medical School, London, UK;* [3]*Alexion Pharmaceuticals Inc., Connecticut, USA*

The intravascular haemolysis of paroxysmal nocturnal haemoglobinuria (PNH) is caused by CD59 deficiency from PNH cells. Eculizumab, a humanized monoclonal antibody to C5, inhibits terminal complement activation. Eleven patients have now received eculizumab (900 mg infusion) on alternate weeks for over a year. Eculizumab is well tolerated with no dose limiting toxicity. The biochemical parameters of haemolysis improve within a week of starting therapy and persist over 12 months. Mean LDH decreased from 3017 U/l over 12 months prior to treatment to 547 U/l over 12 months on treatment ($P = 0.002$). Haemoglobinuria decreased from 3.0 to 0.2 paroxysms/patient-month with a significant improvement in quality of life measures. PNH type III red cells progressively increased during treatment from a mean of 36.7% to 67.1% at 12 months ($P < 0.001$). Transfusion requirement reduced from a median 1.8 units/patient/month prior to eculizumab to 0.2 units/patient/month on eculizumab ($P = 0.001$). Patients with purely haemolytic disease (macroscopic haemoglobinuria with a normal platelet count; $n = 6$) had the best response with cessation of haemolysis and a decrease in transfusions from a median of 2.0–0 units/patient-month. Four patients who continued to receive transfusions have been treated with subcutaneous erythropoietin in addition to eculizumab with improved haemoglobin and transfusion requirement. In most patients both the LDH and bilirubin (under 50 μmol/l) remain slightly elevated, haptoglobins remain undetectable and the reticulocytosis persists indicating a low level of well-compensated, extravascular, haemolysis. Two patients experienced transient breakthroughs in complement activity associated with increased LDH, haemoglobinuria and mild symptoms; these resolved immediately upon next eculizumab dose and further breakthroughs were prevented by a slight decrease in the dosing interval. This study demonstrates that eculizumab is well tolerated, dramatically decreases haemolysis and transfusion requirement as well as improving the symptoms of PNH. The effectiveness of eculizumab has been maintained for over 12 months of therapy and is ongoing in all patients.

6

A genetic interaction between chromosome 8q and the β-globin complex influences the switch from foetal to adult haemoglobin

N. Silver,[1] C. Garner,[2] S. Best,[1] S. Menzel,[1] C. Martin,[1] T. Spector[3] & S. L. Thein[1]

[1]*Department of Haematological Medicine, GKT School of Medicine, King's College Hospital, Denmark Hill, London, UK;* [2]*Department of Medicine and Environmental Health, Science and Policy, University of California, Irvine, USA;* [3]*St Thomas' Twins Register Unit, St Thomas' Hospital, London, UK*

The levels of HbF and F cells (FC) in adults are predominantly genetically controlled, influenced by several factors, including a common sequence variant (C to T) at position – 158 upstream of the Gγ-globin gene, referred to as the *Xmn*1-Gγ polymorphism. About 60% of the FC variance, however, is due to transacting factors. Three loci for FC levels have been mapped, on chromosome Xp22, 6q23, and most recently, on 8q in an extensive Indian kindred. The influence of the 8q quantitative trait locus (QTL) was shown to be conditional on the genotype at the *Xmn*1-Gγ site, suggesting a genetic interaction between the site and the 8q QTL. In an attempt to replicate the chromosome 8q linkage, 319 dizygotic (DZ) twin pairs of European descent were phenotyped for HbF and FC levels and genotyped for a set of 19 markers spanning 56 cM of chromosome 8. The effect of the *Xmn*1-Gγ genotype on the FC levels was estimated by linear regression and the effect of the genotype on the trait values was adjusted. The *Xmn*1-Gγ adjusted FC trait showed higher multipoint lod scores across the 56 cM interval than the unadjusted trait, with a maximum lod score of 2.76. The DZ twin sample was stratified according to the six possible unordered twin pair *Xmn*1-Gγ genotypes and linkage analysis was carried out with each subsample. The linkage between the chromosome 8q QTL and FC levels

Samsung Bioepis Exhibit No. 1027
Page 175

ORIGINAL ARTICLE

Effect of Eculizumab on Hemolysis and Transfusion Requirements in Patients with Paroxysmal Nocturnal Hemoglobinuria

Peter Hillmen, M.B., Ph.D., Claire Hall, M.B., Ch.B., Judith C.W. Marsh, M.B., M.D., Modupe Elebute, M.B., M.D., Michael P. Bombara, B.S., Beth E. Petro, B.S., Matthew J. Cullen, B.Sc., Stephen J. Richards, Ph.D., Scott A. Rollins, Ph.D., Christopher F. Mojcik, M.D., Ph.D., and Russell P. Rother, Ph.D.

ABSTRACT

BACKGROUND

Paroxysmal nocturnal hemoglobinuria (PNH) arises from a somatic mutation of the *PIG-A* gene in a hematopoietic stem cell and the subsequent production of blood cells with a deficiency of surface proteins that protect the cells against attack by the complement system. We tested the clinical efficacy of eculizumab, a humanized antibody that inhibits the activation of terminal complement components, in patients with PNH.

METHODS

Eleven transfusion-dependent patients with PNH received infusions of eculizumab (600 mg) every week for four weeks, followed one week later by a 900-mg dose and then by 900 mg every other week through week 12. Clinical and biochemical indicators of hemolysis were measured throughout the trial.

RESULTS

Mean lactate dehydrogenase levels decreased from 3111 IU per liter before treatment to 594 IU per liter during treatment (P=0.002). The mean percentage of PNH type III erythrocytes increased from 36.7 percent of the total erythrocyte population to 59.2 percent (P=0.005). The mean and median transfusion rates decreased from 2.1 and 1.8 units per patient per month to 0.6 and 0.0 units per patient per month, respectively (P=0.003 for the comparison of the median rates). Episodes of hemoglobinuria were reduced by 96 percent (P<0.001), and measurements of the quality of life improved significantly.

CONCLUSIONS

Eculizumab is safe and well tolerated in patients with PNH. This antibody against terminal complement protein C5 reduces intravascular hemolysis, hemoglobinuria, and the need for transfusion, with an associated improvement in the quality of life in patients with PNH.

From the Department of Haematology, Leeds Teaching Hospitals National Health Science Trust, Leeds, United Kingdom (P.H., C.H., M.J.C., S.J.R.); the Department of Haematology, St. George's Hospital Medical School, London (J.C.W.M., M.E.); and Alexion Pharmaceuticals, Cheshire, Conn. (M.P.B., B.E.P, S.A.R., C.F.M., R.P.R.). Address reprint requests to Dr. Hillmen at the Department of Haematology, Leeds General Infirmary, Great George St., Leeds LS1 3EX, United Kingdom, or at peter.hillmen@panp-tr.northy.nhs.uk.

N Engl J Med 2004;350:552-9.

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Samsung Bioepis Exhibit No. 1027
Page 176

THE MAJOR CLINICAL SIGNS OF PAROXysmal nocturnal hemoglobinuria (PNH) are intravascular hemolysis, venous thrombosis, and hemoglobinuria.[1] The disease arises from a somatic mutation of the *PIG-A* gene in a pluripotent hematopoietic stem cell. *PIG-A* encodes a protein that is essential for the synthesis of glycosylphosphatidylinositol (GPI), a lipid moiety that is embedded in the plasma membrane, where it serves to anchor a wide variety of proteins to the cell surface. The mutant stem cell subsequently expands to form a hematopoietic clone with a deficiency in proteins that are normally attached to the cell surface by the GPI anchor.[2,3] The mature blood cells derived from the hematopoietic clone can have a complete deficiency (type III) or a partial deficiency (type II) of GPI-linked proteins and almost always coexist with residual cells with a normal expression of these proteins (previously identified as type I).

The clinical features of PNH result from the lack of one or more GPI-linked proteins that protect cells from complement-mediated attack. Two such proteins — CD55 and CD59 — are absent from PNH type III erythrocytes, platelets, and other blood cells.[4-6] CD55 regulates early complement activation by inhibiting C3 convertases,[7] whereas CD59 inhibits the assembly of the membrane-attack complex C5b–C9 by interacting with C8 and C9.[4,5] The lack of CD59 is probably responsible for the increased sensitivity of PNH erythrocytes and platelets to complement.[4,8-13]

Eculizumab is a recombinant humanized monoclonal antibody that was designed to block the activation of terminal complement components.[14,15] It binds specifically to the terminal complement protein C5, inhibiting its cleavage into C5a and C5b, thereby preventing the release of the inflammatory mediator C5a and the formation of the cytolytic-pore C5b–C9. Blockade of the complement cascade at C5 preserves the early components of complement that are essential for the opsonization of microorganisms and clearance of immune complexes.[16] In this trial, we investigated whether eculizumab could reduce the incidence of intravascular hemolysis, hemoglobinuria, and transfusion requirements in patients with PNH.

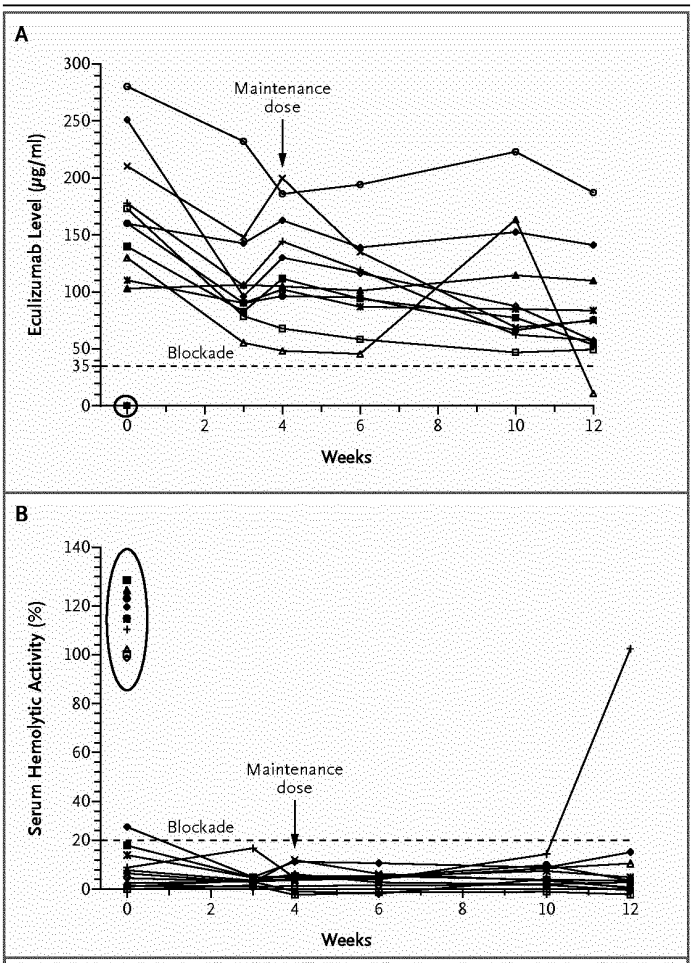


Figure 1. Pharmacokinetic and Pharmacodynamic Analysis of Eculizumab in 11 Patients with Paroxysmal Nocturnal Hemoglobinuria (PNH).

Panel A depicts serum levels of eculizumab during the 12-week treatment period. Eculizumab levels just before a patient's first dose of eculizumab are circled on the x axis. Also shown at time zero are levels of eculizumab one hour after the initial dose. Other data points indicate trough levels of eculizumab for each patient at weeks 3, 4, 6, 10, and 12. The beginning of the 900-mg maintenance dose is indicated by an arrow. The level of eculizumab required to block complement activity (≥35 μg per milliliter) is indicated by a dashed line. Panel B shows hemolytic activity in serum during the 12-week treatment period, as assessed in a presensitized chicken-erythrocyte hemolytic assay. Pretreatment serum hemolytic activity values are circled at time zero. Also shown at time zero are values for hemolytic activity one hour after the initial dose. Other data points indicate trough hemolytic activity at weeks 3, 4, 6, 10, and 12. The dashed line indicates the level of hemolytic activity that is considered to represent complete complement blockade (≤20 percent).

METHODS

PATIENTS

The study was conducted from May through December 2002. Men and women (18 years of age and older) who had received a diagnosis of PNH at least six months earlier, had a detectable GPI-deficient hematopoietic clone, and had received at least four red-cell transfusions in the preceding 12 months were

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eligible. Patients were required to have a negative throat culture for *Neisseria meningitidis* and *N. gonorrhoeae*. All patients were vaccinated against *N. meningitidis* (Mengivac (A+C), Aventis Pasteur) before treatment. One patient had a stroke after consent but never received eculizumab and was excluded. Patients who were taking stable doses of immunosuppressive drugs (e.g., cyclosporine), warfarin, and iron supplements were permitted to continue them.

The trial was approved by the local research ethics committee and was performed according to the International Conference on Harmonisation and Good Clinical Practice Standards. Eleven patients gave written informed consent and were treated with eculizumab.

TREATMENT SCHEDULE

Patients received infusions of 600 mg of eculizumab weekly for four weeks, followed one week later by a 900-mg dose and then by a dose of 900 mg every other week through week 12.

INVESTIGATIONS

In this open-label pilot study, we obtained data on the pharmacokinetics, pharmacodynamics, and immunogenicity of eculizumab and observed its clinical effects by measuring the following: lactate dehydrogenase, haptoglobin, bilirubin, and hemoglobin levels; reticulocyte counts; the proportion of GPI-deficient cells, as assessed by flow cytometry[17]; the rate of transfusion with packed red cells; the rate of occurrence of hemoglobinuria (assessed by daily comparison of the first morning urine sample with a standardized color chart before and during treatment); and the quality of life, as reflected by the scores on the European Organization for Research and Treatment of Cancer (EORTC) QLQ-C30 instrument. The trigger for transfusion during the study period remained unchanged for each patient, as compared with their care before entry into the study: patients received blood transfusions when they had symptoms resulting from anemia.

Assessment of the safety of eculizumab included ascertainment of treatment-related adverse events, electrocardiography, and routine laboratory tests (e.g., serum chemical analyses and complete blood counts).

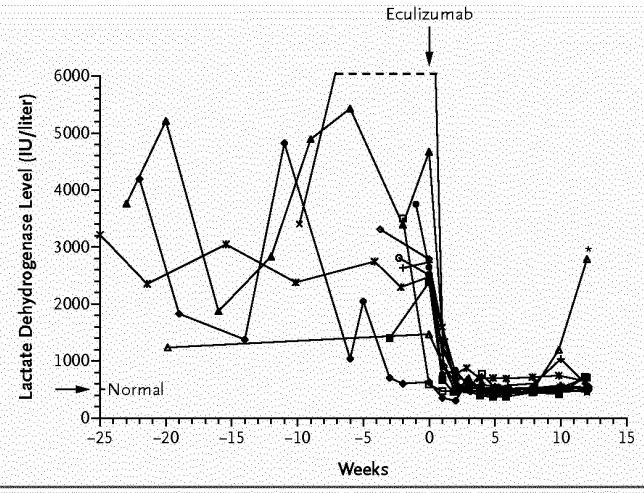


Figure 2. **Analysis of Lactate Dehydrogenase Levels, a Biochemical Indicator of Hemolysis, in 11 Patients with Paroxysmal Nocturnal Hemoglobinuria up to 25 Weeks before and during 12 Weeks of Eculizumab Treatment.**
The first dose of eculizumab is indicated by an arrow, as is the upper limit of the normal range of lactate dehydrogenase at the Leeds Teaching Hospital. The data point identified at week 12 by the asterisk represents a reading that was obtained from a duplicate serum sample since the original sample was lost. The dashed line represents off-scale points from one patient with a peak value of 12,100 IU per liter.

ASSAY METHODS

The pharmacokinetics of eculizumab were determined with an enzyme-linked immunosorbent assay that detects both free and C5-bound eculizumab.[15] The pharmacodynamics of eculizumab were determined by measuring the capacity of the patient's serum to lyse chicken erythrocytes in a standard total human serum-complement hemolytic assay.[18] The presence or absence of antibodies against eculizumab was assessed by an enzyme-linked immunosorbent assay.[19]

STATISTICAL ANALYSIS

Biochemical values were compared with the use of a paired Student's t-test, quality-of-life measurements with the use of a mixed-effect analysis of covariance, the median rate of transfusions with the use of a Wilcoxon signed-rank test, and the comparison of the number of days with paroxysms with the use of Fisher's exact test.

The corresponding author and the sponsor were jointly responsible for the design of this trial and the development of the protocol. Data were collected and analyzed by a clinical research organization, Kendle International, which maintained the trial data base and provided statistical support. The manuscript was prepared by the corresponding author, with substantial review and comments by the other authors and the sponsor. Final decisions on

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the content of the manuscript rested with the corresponding author in consultation with the other authors. All authors had access to the primary data.

RESULTS

DEMOGRAPHIC CHARACTERISTICS

Six men and five women (median age, 48 years; range, 21 to 67) with a median duration of PNH of 8.6 years (range, 1.7 to 37.4) participated in the trial. Five of the patients had platelet counts at base line of less than 150,000 per cubic millimeter. Eight patients had previously received a diagnosis of aplastic anemia, two were concomitantly receiving cyclosporine for aplastic anemia, and six were receiving warfarin.

SAFETY

All patients completed the 12-week study. There were no deaths or thrombotic events, and all patients subsequently entered a 12-month extension study. Each patient reported one or more adverse events during the trial. Events reported by three patients included headache and upper respiratory tract infection. Events reported by two patients included influenza-like symptoms, rigors, dizziness, nausea, nasal congestion, and joint aches. None of these events were attributed to the study medication. Serious adverse events occurred in two patients. The first was hospitalized with a viral chest infection. The second reported nausea, vomiting, and headache after the first infusion, with dizziness and shivering the following day. The patient was hospitalized overnight, and subsequent infusions were well tolerated.

PHARMACOKINETICS, PHARMACODYNAMICS, AND IMMUNOGENICITY OF ECULIZUMAB

Peak and trough levels of eculizumab were well above 35 μg per milliliter from one hour after the first dose through the completion of the 600-mg weekly dose period (Fig. 1A). In 10 patients, serum trough levels of eculizumab remained above 35 μg per milliliter for the entire study.

The hemolytic activity of serum from these 10 patients was completely blocked (less than 20 percent in the chicken–red-cell assay) for essentially the entire treatment period (Fig. 1B). In 1 of the 11 patients, the trough level of eculizumab fell below 35 μg per milliliter at week 12 and serum hemolytic activity returned. In no case were antibodies against eculizumab detected.

BIOCHEMICAL INDICATORS OF HEMOLYSIS

Levels of lactate dehydrogenase in serum were markedly elevated in all patients before eculizumab treatment. Mean (±SD) lactate dehydrogenase levels fell from 3111±598 IU per liter during the 12 months before enrollment to 594±32 IU per liter (normal range, 150 to 480) during treatment ($P=0.002$) (Fig. 2).

The decrease in lactate dehydrogenase began after a single dose of eculizumab in all patients. Lactate dehydrogenase levels remained within or just above the normal range for the duration of the study (Fig. 2). In the one patient in whom eculizumab levels fell below 35 μg per milliliter at week 12 (Fig. 1A), hemolytic activity returned (Fig. 1B) and lactate dehydrogenase levels increased transiently (Fig. 2).

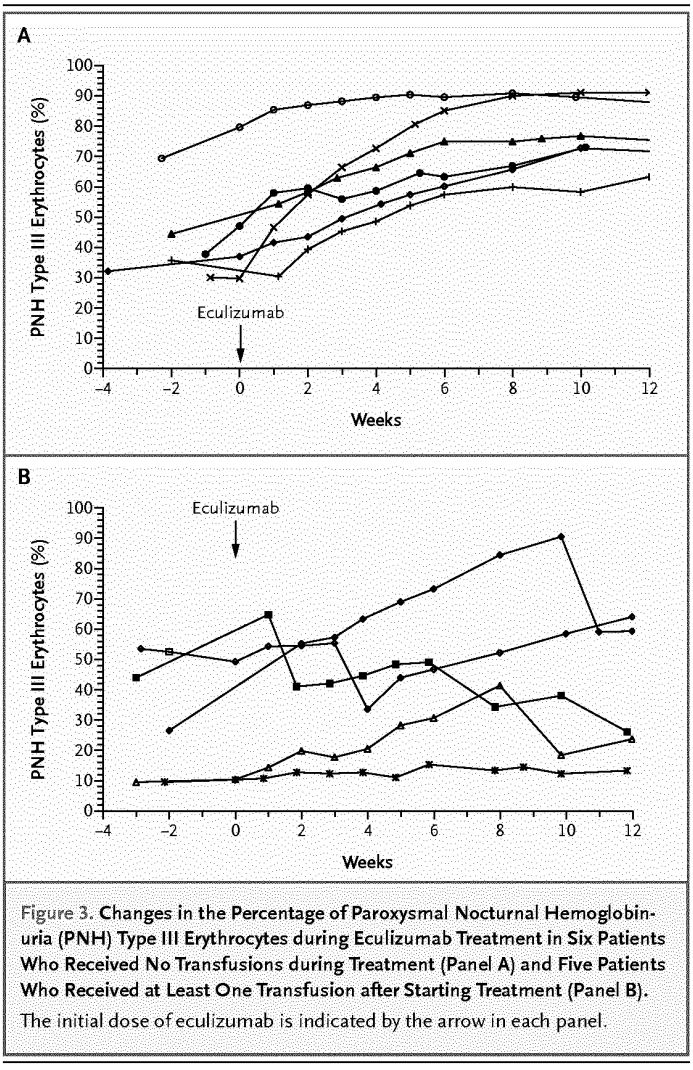


Figure 3. **Changes in the Percentage of Paroxysmal Nocturnal Hemoglobinuria (PNH) Type III Erythrocytes during Eculizumab Treatment in Six Patients Who Received No Transfusions during Treatment (Panel A) and Five Patients Who Received at Least One Transfusion after Starting Treatment (Panel B).** The initial dose of eculizumab is indicated by the arrow in each panel.

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The dosing frequency was increased from 900 mg every 14 days to 900 mg every 12 days, reestablishing complete complement blockade during the ongoing 12-month extension study (data not shown).

Haptoglobin became detectable in the serum of 5 of the 11 patients after two weeks of eculizumab treatment but returned to undetectable levels soon thereafter (data not shown). Bilirubin levels were also elevated in most patients at base line and did not change significantly during treatment (data not shown).

EFFECT ON PNH CLONES

Type III erythrocytes are highly sensitive to lysis by complement and as a result have a short life span. In our study, the percentage of type III erythrocytes increased significantly from a mean of 36.7±5.9 percent before treatment to 59.2±8.0 percent at the end of 12 weeks of treatment (P=0.005) (Fig. 3). The increase was particularly consistent in six patients who remained transfusion-independent during the study (Fig. 3A). In patients who received transfusions during the study, sudden drops in the proportion of type III cells were seen as the transfused red cells diluted the type III population (Fig. 3B). There were no significant changes in the percentages of PNH type III neutrophils, monocytes, or platelets during treatment with eculizumab; in most cases, these percentages were 90 to 100 percent before the study (data not shown).

TRANSFUSION REQUIREMENTS, HEMOGLOBIN LEVELS, AND RETICULOCYTE COUNTS

During the year preceding enrollment, the range of red-cell transfusions received by the 11 patients was 12 to 55 units, whereas during the three months of the study, the range was 0 to 8 units. Before eculizumab treatment, the mean and median transfusion rates were 2.1 and 1.8 units per patient per month, respectively (Table 1). These transfusion rates decreased to 0.6 and 0.0 unit per patient per month, respectively, during the three months of treatment with eculizumab (P=0.003). Hemoglobin levels did not increase significantly during the treatment period, although hemoglobin values in six patients stabilized without transfusions (Table 1). Similarly, the numbers of reticulocytes remained relatively constant during eculizumab treatment.

HEMOGLOBINURIA

Our patients recorded the color of their urine each morning using a color chart designed to assess the

Table 1. **Transfusion Rates, Hemoglobin Levels, and Reticulocyte Counts 12 Months before and after 3 Months of Eculizumab Treatment.**

Patient No.	12 Mo before Eculizumab Treatment				After 3 Mo of Eculizumab Treatment			
	Transfusions		Hemoglobin	Reticulocytes	Transfusions		Hemoglobin	Reticulocytes
	no. of units	*rate**	*g/dl*	*×10⁻³/mm³*	*no. of units*	*rate†*	*g/dl*	*×10⁻³/mm³*
1	22	1.8	10.3	77.5	2	0.7	10.0	100.7
2	23	1.9	8.3	200.0	8	2.9	8.8	182.6
3	20	1.6	10.1	169.5	0	0.0	10.7	175.9
4	28	2.3	9.3	282.0	0	0.0	9.4	333.3
5	12	1.0	11.9	96.3	2	0.7	10.6	121.8
6	14	1.2	9.8	346.8	0	0.0	10.6	259.0
7	34	2.8	12.8	100.6	0	0.0	13.5	166.8
8	21	1.7	9.5	164.5	0	0.0	9.8	239.6
9	55	4.5	10.7	138.0	3	1.1	11.4	285.8
10	41	3.4	8.5	108.7	5	1.8	8.8	140.1
11	14	1.2	8.5	91.4	0	0.0	10.0	97.4
Median‡		1.8				0.0		
Mean		2.1	10.0	161.4		0.6	10.3	191.2

* The rate (in units per month) was calculated as (number of units÷365 days)×30.
† The rate (in units per month) was calculated as (number of units÷84 days)×30.
‡ P=0.003 for the change in the median rate of transfusion by the Wilcoxon signed-rank test.

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degree of hemoglobinuria (Fig. 4A) during both the 2-to-4-week screening period and the 12-week treatment period. Paroxysms of hemoglobinuria were prospectively defined as dark-colored urine with a colorimetric level of 6 or more. In nine patients for whom urine scores were assessed, the mean incidence of paroxysms was reduced from 2.9 days to 0.12 day per patient per month ($P<0.001$) (Fig. 4B).

QUALITY OF LIFE

The quality of life was assessed with the use of the EORTC QLQ-C30 instrument. When responses at base line were compared with responses during 12 weeks of eculizumab treatment, there were significant improvements in the domains of global health status ($P=0.02$), physical functioning ($P<0.001$), emotional functioning ($P<0.001$), cognitive functioning ($P=0.002$), fatigue ($P<0.001$), dyspnea ($P=0.002$), and insomnia ($P=0.049$) (Table 2).

DISCUSSION

Patients with PNH have chronic, often disabling symptoms of fatigue and intermittent episodes of dysphagia, abdominal pain, and hemoglobinuria. These symptoms are thought to be related to the intravascular destruction of PNH type III erythrocytes, which are deficient in complement inhibitors, by autologous complement. The hemolytic anemia frequently renders the patients transfusion-dependent. In addition, patients have an extremely high risk of potentially life-threatening thrombosis, particularly thrombosis of the hepatic and cerebral veins. Approximately 50 percent of patients with PNH die of the disease; the median duration of survival after diagnosis is 10 years.[1]

We found that the defect in the membrane-bound inhibitor of terminal complement components in PNH was ameliorated by the administration of eculizumab. This antibody specifically prevents cleavage of C5, which is necessary for assembly of the membrane-attack complex. Blockade of terminal complement components presumably prolongs the survival of type III erythrocytes (since there was no simultaneous increase in reticulocytes), which are highly sensitive to lysis by complement, thereby increasing the proportion of these cells in the blood and reducing signs of hemolysis in most patients. In some patients, the percentage of type III erythrocytes increased to more than 80 percent of the total erythrocyte population. This interpretation of the mechanism of action of eculizumab is consistent with the report of an asymptomatic patient with PNH who had more than 80 percent type III erythrocytes and a concomitant deficiency in the terminal complement protein C9.[20] Thus, inhibition of the assembly of C5b–C9 by an antibody or by a congenital deficiency of a terminal component of the complement system can protect type III erythrocytes from complement-mediated lysis.

The long-term effects of protecting PNH type III erythrocytes from complement are not known. For example, will removing the negative pressure

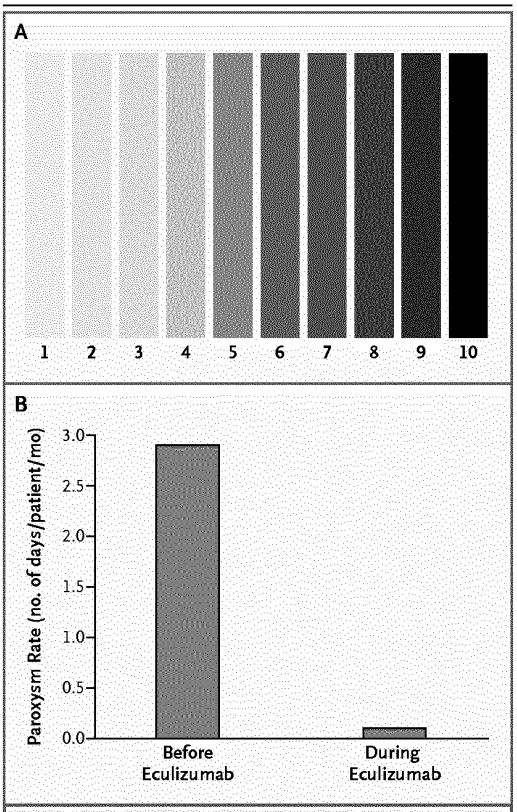


Figure 4. Incidence of Paroxysms during Eculizumab Treatment.

Panel A shows a urine color scale devised to monitor the incidence of paroxysms of hemoglobinuria in patients with paroxysmal nocturnal hemoglobinuria before and during treatment. A paroxysm was prospectively defined as a urine score of 6 or greater in this study. Panel B depicts the change in the paroxysm rate (defined as the mean number of days in paroxysm per patient per month) in nine patients 1 month before and during 12 weeks of eculizumab therapy. Pretreatment data on paroxysms were inadvertently not collected for two patients, and these two were therefore excluded from the analysis.

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Table 2. **Change in the Quality of Life during Eculizumab Treatment.***

Domain	Mean Base-Line Score†	Change from Base Line‡§	P Value§
Global health status	56.1	13.7	0.02
Physical functioning	70.9	13.0	<0.001
Emotional functioning	70.5	12.7	<0.001
Cognitive functioning	77.3	11.8	0.002
Fatigue	47.5	−15.3	<0.001
Dyspnea	39.4	−12.4	0.002
Insomnia	30.3	−10.8	0.049

* The quality of life was assessed with the European Organization for Research and Treatment of Cancer QLQ-C30 instrument.
† Numbers represent mean values of linearly transformed scores.
‡ Values for change from base line represent least-square means. A positive value indicates an improvement in the score for global health status, physical functioning, emotional functioning, and cognitive functioning, whereas a negative value indicates an improvement in the score for fatigue, dyspnea, and insomnia.
§ Values are from a mixed analysis-of-covariance model with visit as a fixed effect, patient as a random effect, and base line as a covariate.

on type III hematopoietic cells alter the rate of expansion of the PNH clone? What might occur in a patient with an increased population of PNH type III erythrocytes if treatment with eculizumab is stopped? Two of the patients who entered the eculizumab extension study had transient breakthroughs (lasting two to three days) in complement blockade until the dosing interval was adjusted. Both patients had hemoglobinuria with mild symptoms, but the episodes were not life threatening and were easily managed (data not shown). Definitive answers to these questions will require further study.

In this trial, lactate dehydrogenase levels declined rapidly and remained reduced as long as the serum level of eculizumab exceeded 35 μg per milliliter. The importance of maintaining this level of antibody was demonstrated in a single patient, in whom the eculizumab level transiently dropped below 35 μg per milliliter at week 12, resulting in a return of serum complement activity and an increase in lactate dehydrogenase levels. Subsequent administration of eculizumab reestablished complement blockade and rapidly reduced lactate dehydrogenase levels. Interestingly, lactate dehydrogenase levels were reduced in most patients to just above the upper limit of normal. The slightly elevated levels of this enzyme during treatment with eculizumab could reflect persistent, low-level C3b-mediated extravascular hemolysis or, possibly, undefined mechanisms of hemolysis that are unrelated to complement.

We also found that eculizumab treatment significantly reduced transfusion requirements, even though the levels of hemoglobin did not change significantly. However, the hemoglobin level in an individual patient before study entry was artificially maintained as a result of the transfusion of normal red cells. The transfused red cells survive far longer than PNH cells. Therefore, the stabilization of hemoglobin levels with a reduced need or no need for transfusion is a result of the protection of PNH red cells from complement-mediated lysis by eculizumab.

The decrease in transfusion requirements was most apparent in the six patients without a clinically significant degree of bone marrow failure (as defined by a normal platelet count). All but one of these patients were transfusion-independent during the study and remained so during an extension study. The remaining patient received a single 3-unit transfusion during the study, as compared with the receipt of 55 units in the 12 months preceding enrollment.

There was a rapid improvement in the quality of life during eculizumab therapy, as measured by the EORTC QLQ-C30. These clinical observations support the hypothesis that many of the important coexisting clinical conditions in patients with PNH are directly related to chronic and acute episodes of hemolysis, possibly through the scavenging of nitric oxide by plasma free hemoglobin.[21-25]

Eculizumab was safe and well tolerated during this open-label pilot study. The adverse events reported by patients were similar in type and frequency to those reported with either eculizumab or placebo in other controlled trials. All patients are currently participating in a one-year extension study in which the drug continues to be well tolerated. Furthermore, the rates of intravascular hemolysis, as measured by lactate dehydrogenase levels and hemoglobinuria, remain reduced in all patients, with 5 of 11 patients having been transfusion-independent for at least one year since starting eculizumab treatment.

The *PIG-A* mutation in patients with PNH causes deficiencies in the membrane-bound complement inhibitors CD55 and CD59, resulting in intravascular hemolysis.[2,3] However, patients who have genetic deficiencies in the surface expression of CD55 (Inab phenotype) with normal levels of CD59 have no clinical signs of hemolysis.[8,9] Conversely, a pa-

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tient with a genetic deficiency in the expression of CD59 but normal levels of CD55 had symptoms indistinguishable from those of PNH.[10,11] Therefore, the somatic mutation in the *PIG-A* gene that causes the deficiency of the membrane-bound terminal complement inhibitor CD59 is critical to the pathogenesis of PNH. We found that terminal complement inhibition with eculizumab ameliorated the untoward effects of this deficiency.

In summary, eculizumab appears to enhance the survival of type III PNH erythrocytes, improving the quality of life and reducing the extent of hemolysis, hemoglobinuria (the clinical hallmark of PNH), and the need for blood transfusions in patients with PNH. This study confirms that terminal complement activation is the key mediator of erythrocyte destruction in PNH.

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Dr. Hillmen reports serving as a consultant to Alexion Pharmaceuticals and receiving grant support from the company; Drs. Rollins, Mojcik, and Rother, Mr. Bombara, and Ms. Petro report having equity ownership in Alexion Pharmaceuticals; and Drs. Rollins and Rother have assigned to Alexion Pharmaceuticals their inventions made as employees of the company and have received no royalties from Alexion for these inventions. Dr. Rollins receives royalties for inventions he made before becoming an employee of Alexion.

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β-glucan released in the BM by Mϕ functions to prime the CR3 of HSPC that have been tethered to the iC3b deposited onto BM cells, thereby enhancing the normal rate of HSPC expansion and differentiation. The oral WGP preparation is currently under study as a possible therapeutic to be given to individuals at risk for exposure to harmful radiation as the result of a nuclear power plant accident or nuclear terrorism. These data indicate an important role in hematopoiesis of both the C3a–C3aR axis and the iC3b–CR3 receptor axis, and do not exclude the participation of other components or receptors.

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Keywords: Bone marrow recovery; β-Glucan

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Eculizumab, a C5 complement-blocking antibody, abolishes hemolysis and reduces transfusion dependency in patients with paroxysmal nocturnal hemoglobinuria (PNH)

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Paroxysmal nocturnal hemoglobinuria (PNH) is characterized by intravascular hemolysis, venous thrombosis and is associated with aplastic anemia. The complement-mediated hemolysis is due to the deficiency of complement-regulatory proteins on the surface of PNH red cells, particularly the terminal complement inhibitor CD59. Eculizumab is a humanized monoclonal antibody that binds to C5 and inhibits terminal complement activation. Eleven transfusion-dependent PNH patients have completed an open-label pilot study of eculizumab. Patients received 600 mg eculizumab by intravenous infusion over 30 min weekly for four doses, followed by 900 mg 1 week later, and then 900 mg every other week for the remainder of the trial. All 11 patients completed the study and are currently participating in an open-label extension study. Eculizumab was well tolerated with no patients reporting a serious adverse event. The biochemical parameters of hemolysis dramatically improved or normalized for all patients within a week of starting therapy. Mean LDH decreased from 3111 ± 598 U/l during the 12 months prior to treatment to 594±32 U/l following treatment ($P = 0.002$) while mean AST decreased from 76 ± 16 U/l at baseline to 28 ± 4 U/l following treatment ($P = 0.032$). Paroxysms, as defined by episodes of hemoglobinuria on a urine colorimetric scale, decreased by 96% ($P < 0.001$). In addition, PNH type III red cells increased 65% from 36.7 ± 5.9 to 59.2 ± 8.0% during the treatment period ($P = 0.005$), presumably due to a prolongation of PNH red cell survival. Transfusions were significantly reduced in the 3 months of eculizumab compared to the previous 12-month historical data from 2.03 to 0.65 units per patient per month ($P = 0.023$). Quality of life (EORTC QLC30) improved significantly post-eculizumab compared to pre-eculizumab. There was no change in the proportion of PNH neutrophils, monocytes or platelets in any of the patients while on eculizumab. No episodes of thrombosis occurred in these 11 patients although follow-up is short and 4 of 6 patients are on warfarin. In summary, the results from this pilot study demonstrate that eculizumab was well tolerated, decreased hemolysis and improved symptoms in PNH patients. This study suggests that blocking complement at C5 is an effective therapeutic strategy for hemolytic PNH resulting in control of hemolysis and a reduction in transfusion dependency.

Keywords: Paroxysmal nocturnal hemoglobinuria (PNH); C5

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DX-88 a recombinant inhibitor of human plasma kallikrein. Efficacy and safety in hereditary and acquired angioedema

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DX-88 is a recombinant inhibitor of plasma kallikrein. It was designed as a Kunitz domain protein and selected by phage display to be highly specific for human plasma kallikrein. Safety and efficacy of DX-88 in reverting symptoms in patients with hereditary and acquired angioedema (HAE and AAE) has been tested.

The study was designed to have nine different HAE/AAE patients treated with DX-88 for a non-lifethreatening angioedema. The three ascending dose regimens, (10, 40, and 80 mg) given as a short intravenous infusion, were used. Time to relief of symptoms, time to resolution and adverse events have been recorded. The following biochemical parameters have been assessed: plasma levels of C1INH and kallikrein function, plasma concentrations of C1INH and C4, cleavage of HK pharmacokinetic of DX-88.

Seven patients with HAE and two with AAE (five women and four men, age range 31–67 years old) have been treated. Angioedema was localized to the face (four patients), to the hands (one patients) to the genitals (one patient) and

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Oral Communication

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Eculizumab, a C5 complement blocking antibody, is the first therapy to reduce transfusion requirements in patients with paroxysmal nocturnal haemoglobinuria (PNH)

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Paroxysmal nocturnal haemoglobinuria (PNH) is characterized by intravascular haemolysis and venous thrombosis due to the unopposed effect of complement on PNH red cells and platelets, respectively. Eculizumab is a humanized monoclonal antibody that binds C5 and inhibits terminal complement activation. Eleven transfusion-dependent PNH patients have completed a 3-month pilot study receiving 600 mg eculizumab by intravenous infusion over 30 min weekly for four doses and then 900 mg every other week. Adverse events were relatively minor and self-limiting. The biochemical parameters of haemolysis dramatically improved for all patients within a week. Mean LDH decreased from 3089 ± 599 U/L before to 597 ± 32 U/L after treatment ($P = 0.002$) and AST from 77 ± 20 to 26 ± 3 U/L ($P = 0.04$). Episodes of haemoglobinuria reduced by 90% ($P < 0.001$). Quality of life (EORTC QLC30) improved significantly post-eculizumab compared to pre-eculizumab. One patient began treatment during a severe acute haemolytic episode with almost black urine and within 24 h there was a complete resolution of her haemoglobinuria and symptoms. Transfusions for all patients were significantly reduced in the 3 months of eculizumab compared with the previous 12 months from 2.2 to 0.7 units/month ($P = 0.003$). The reduction in transfusions was most dramatic in the seven patients without thrombocytopenia from 2.3 to 0.2 units/month ($P = 0.001$). There was an increase in PNH type III red cells from a mean of 35.6% pre-eculizumab to 58.7% post-eculizumab ($P = 0.005$) due to their protection from complement-mediated lysis and the reduction in transfusions. There was no change in the proportion of PNH neutrophils, monocytes or platelets. All patients have continued on eculizumab in an extension study. In summary, eculizumab is well tolerated, decreases haemolysis, improves symptoms and significantly reduces transfusions in PNH patients. Eculizumab appears to be the first effective therapy for haemolysis in PNH and merits further development. This is the first trial of an anti-complement antibody therapy in PNH. The responses were remarkable both in reduction in haemolysis and transfusions and in the improvement in quality of life. Thus it is the first effective therapy for haemolysis in PNH and would also be expected to reduce the risk of thrombosis (this study could not address this). The trial was performed at two centres in the UK only.

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The induction of tolerance to RhD in HLA DR15 transgenic mice

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The RhD protein is a clinically important red cell alloantigen that induces the production of anti-D in RhD-negative individuals. Because of the immunogenicity of RhD, all blood donations are typed and matched for this antigen, and RhD-negative women are managed for the prevention of haemolytic disease of the newborn with prophylactic anti-D immunoglobulin. Understanding the mechanisms by which helper T-cells control the antibody response to RhD would enable the design of alternative strategies such as active toleration. We have shown that CD4+ helper T cells from alloimmunized donors can proliferate and produce both IFN-gamma and IL-4 in response to synthetic peptides derived from the RhD sequence, indicating a Th0 balanced cytokine response. The responses are HLA class II restricted, with HLA-DR15 being over-represented in good responders. Certain peptides are immunodominant, and induce proliferative responses in most alloimmunized donors. These peptides are candidates for the induction of tolerance to the RhD protein by the mucosal route. On the other hand, some peptides from the RhD protein preferentially induce IL-10 production by putative Tr1 regulatory cells *invitro*, and these peptides inhibit T-cell proliferation in response to stimulation with purified RhD protein *in vitro*. These peptides may be used to induce tolerance by the parenteral route. We have used a HLA-DR15 transgenic mouse model to show that T-cells from mice immunized with the RhD protein proliferate in response to purified RhD protein and to RhD peptides, and serum antibodies specific for human erythrocytes are also produced. Mucosal administration of immunodominant RhD peptides, or parenteral administration of IL-10 inducing peptides prior to immunization with RhD protein, inhibits both T-cell proliferation and antibody production. These results suggest that synthetic RhD peptides can induce tolerance to the RhD protein and may be of value in the prevention of immunization in RhD-negative individuals.

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A humanized monoclonal antibody against vWF A1 domain inhibits vWF:RiCof activity and platelet adhesion in human volunteers

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AJW200 (Ajinomoto), a humanized IgG4 monoclonal antibody directed against the A1 domain of von Willebrand factor (vWF), specifically inhibits vWF:platelet GPIb receptor interactions. At

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Eculizumab Could Be Paroxysmal Nocturnal Hemoglobinuria Breakthrough

Dec 13, 2002

Jane Salodof MacNeil

Dec. 13, 2002 (Philadelphia) - Preliminary results from an ongoing open-label phase Ib clinical trial suggest an experimental drug called eculizumab could be the first to alleviate symptoms of paroxysmal nocturnal hemoglobinuria (PNH).

Data on the first 11 patients show that three months of eculizumab therapy reduced the need for blood transfusions by 68%. An 81% reduction in biochemical parameters of hemolysis was also reported, and duration of clinical paroxysms went down by 90% from 2.1 days per patient per month to 0.2.

"With leukemia trials you have to wait months and years to see if there's a benefit. You knew immediately with this drug," principal investigator Peter Hillmen, MB, ChB, PhD, told Medscape. "[The patients] came in the next week, and they were fine."

Dr. Hillmen, a clinical hematologist at The General Infirmary at Leeds in the U.K., presented the results here at the American Society of Hematology annual meeting.

The paper was chosen because eculizumab would be the first targeted therapy with relevance to PNH, according to Russell Ware, MD, PhD, of Duke University Medical Center, co-chair of the session on acquired disorders.

Dr. Ware called the work "very exciting" in an interview with Medscape, but cautioned, "There's still a lot of work to be done. They need to study dosing, side effects — both short and long term — and risks as well as benefits to patients."

An acquired genetic disorder, PNH occurs in roughly one in 200,000 people, according to Dr. Hillmen.

Patients with PNH are deficient in the PIG-A enzyme and consequently their cells cannot protect themselves from complements in the blood. As a result, red blood cells are prone to lysis, producing blood in the person's plasma and urine, which often becomes dark red.

Patients typically become anemic, suffer significant fatigue, and have a high risk of dying from thrombosis. They are usually treated with steroids such as prednisone, immunosupressive drugs, and blood transfusions.

Eculizumab is a long-acting C5 inhibitor that Alexion Pharmaceuticals Inc. of Cheshire, Connecticut, is also testing in rheumatoid arthritis. Inhibition of complements 5-9 is associated with slightly higher risk of meningococcal infection, according to Dr. Hillmen. Only one case has been observed among 500 patients who have taken the drug so far, he said, including the rheumatoid arthritis trials.

"That's pretty good, and yet a case of meningitis might not be trivial," Dr. Ware noted.

The PNH trial was conducted with Alexion support at two sites in the U.K. Patients received 600 mg of eculizumab each week for four weeks, after which the dose was switched to 900 mg every two weeks.

Ten patients have completed the 12-week treatment, and all chose to enter a 12-month extension trial. Another trial was at 10 weeks when the data was reported. All patients had at least four transfusions in the 12 months prior to the trial (average, 9 transfusions). The seven patients whose platelets levels were above 100 had the best response. Those with platelets levels below 100 did not improve as dramatically, Dr. Hillmen said, suggesting the drug is less effective in patients with bone marrow failure.

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Quality-of-life measures showed improvement in physical functioning, global health status, fatigue, and dyspnea parameters. Dr. Hillmen said some patients in the study had been so debilitated by fatigue that they had not worked for as long as 30 years.

"He overtook me on the stairs last week," Dr. Hillmen said of one of the most debilitated patients prior to treatment. "It had that dramatic an effect,"

The investigators reported that the drug was well tolerated. Muscle and joint aches and pains, upper respiratory infection, and headache were the most frequent adverse events.

A spokesman for Alexion Pharmaceuticals told Medscape that the company plans to work with the U.S. Food and Drug Administration's orphan drug program to design what will probably be a phase II/III trial.

ASH 44th Annual Meeting: Abstract 154. Presented Dec. 9, 2002.

Reviewed by Gary D. Vogin, MD

Jane Salodof MacNeil is a freelance writer for Medscape.

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Immune-Mediated Hemolytic Anemia

Wendell F. Rosse, Peter Hillmen, and Alan D. Schreiber

Hemolytic anemia due to immune function is one of the major causes of acquired hemolytic anemia. In recent years, as more is known about the immune system, these entities have become better understood and their treatment improved. In this section, we will discuss three areas in which this progress has been apparent.

In Section I, Dr. Peter Hillmen outlines the recent findings in the pathogenesis of paroxysmal nocturnal hemoglobinuria (PNH), relating the biochemical defect (the lack of glycosylphosphatidylinositol [GPI]-linked proteins on the cell surface) to the clinical manifestations, particularly hemolysis (and its effects) and thrombosis. He discusses the pathogenesis of the disorder in the face of marrow dysfunction insofar as it is known. His major emphasis is on innovative therapies that are designed to decrease the effectiveness of complement activation, since the lack of cellular modulation of this system is the primary cause of the pathology of the disease. He recounts his considerable experience with a humanized monoclonal antibody against C5, which has a remarkable effect in controlling the manifestations of the disease. Other means of controlling the action of complement include replacing the missing modulatory proteins on the cell surface; these studies are not as developed as the former agent.

In Section II, Dr. Alan Schreiber describes the biochemistry, genetics, and function of the Fcγ receptors and their role in the pathobiology of autoimmune hemolytic anemia and idiopathic thrombocytopenic purpura due to IgG antibodies. He outlines the complex varieties of these molecules, showing how they vary in genetic origin and in function. These variations can be related to three-dimensional topography, which is known in some detail. Liganding IgG results in the transduction of a signal through the tyrosine-based activation motif and Syk signaling. The role of these receptors in the pathogenesis of hematological diseases due to IgG antibodies is outlined and the potential of therapy of these diseases by regulation of these receptors is discussed.

In Section III, Dr. Wendell Rosse discusses the forms of autoimmune hemolytic anemia characterized by antibodies that react preferentially in the cold–cold agglutinin disease and paroxysmal cold hemoglobinuria (PCH). The former is due to IgM antibodies with a common but particular structure that reacts primarily with carbohydrate or carbohydrate-containing antigens, an interaction that is diminished at body temperature. PCH is a less common but probably underdiagnosed illness due to an IgG antibody reacting with a carbohydrate antigen; improved techniques for the diagnosis of PCH are described. Therapy for the two disorders differs somewhat because of the differences in isotype of the antibody. Since the hemolysis in both is primarily due to complement activation, the potential role of its control, as by the monoclonal antibody described by Dr. Hillmen, is discussed.

I. Paroxysmal Nocturnal Hemoglobinuria: Current Understanding of the Biology of PNH

*Peter Hillmen, PhD**

Paroxysmal nocturnal hemoglobinuria (PNH)[1,2] is characterized by chronic intravascular hemolysis that is punctuated by episodes, or paroxysms, during which there is a marked increase in the intensity of hemolysis with macroscopic hemoglobinuria. The other elements of the clinical triad of PNH are venous thrombosis and an association with aplastic anemia. Recent therapeutic developments promise to radically alter the natural history of hemolytic PNH.

PNH is unique because it is an acquired hemolytic anemia due to an intrinsic red cell defect. In 1970 Oni et al first demonstrated that PNH red cells are monoclonal and that the remaining normal red cells are

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polyclonal. It was clear by the 1980s that the PNH cells were deficient in a large number of cell surface antigens but it was unclear how a single mutation could lead to the deficiency of such a variety of antigens nor how this resulted in the hemolysis characteristic of PNH.

Glycosylphosphatidylinositol-Linked Antigens and PNH

In the 1980s it became clear that a variety of antigens were attached to the cell membrane by a glycolipid structure. This structure is highly preserved throughout evolution with the same basic "backbone" consisting of a phosphatidylinositol, a single glucosamine, three mannoses and an ethanolamine—a glycosylphosphatidylinositol (GPI) structure. The antigens missing from PNH cells are all GPI-anchored to the cell membrane and thus a single mutation disrupting GPI biosynthesis would result in the PNH phenotype. The biosynthetic defect in PNH always affects the transfer of glucosamine from UDP-*N*-acetyl glucosamine onto phosphatidyl inositol. Kinoshita and his group first cloned the *pig-a* gene, which was subsequently found to be mutated in all cases of PNH reported to date.[3-5]

Intravascular Hemolysis in PNH

The work of both Ham and Dacie in the 1930s first revealed that the hemolysis in PNH was due to the effect of a serum factor on abnormal PNH red cells. Rosse proceeded to show that this factor was complement that when activated led to the intravascular hemolysis of PNH red cells. The characteristic symptoms of PNH—abdominal pain, dysphagia, erectile failure and severe lethargy—can be attributed to the intense intravascular hemolysis and the resulting free plasma hemoglobin. This appears to be due to the absorption of nitric oxide by free hemoglobin and since nitric oxide is critical for smooth muscle function this results in the symptoms described (see below).

The functions of the GPI-linked antigens are extremely varied. At least two are important in the control of complement. Decay accelerating factor (DAF or CD55) controls the early part of the complement cascade by regulating the activity of the C3 and C5 convertases. Thus CD55 deficiency initially appeared to explain the sensitivity of PNH red cells to complement. However, the observation that individuals with inherited CD55 deficiency (Inab- phenotype) did not suffer from hemolysis proved that deficiency of CD55 does not cause the hemolysis in PNH. Membrane inhibitor of reactive lysis (MIRL or CD59) was identified in 1989 and is also GPI-linked. CD59 inhibits terminal complement by preventing the incorporation of C9 onto C5b-8 and therefore preventing the formation of the membrane attack complex (MAC). In 1990 an individual with inherited isolated deficiency of CD59 was described with many features similar to classic PNH, such as intravascular hemolysis with hemoglobinuria and thrombosis of the cerebral veins.[6] Thus CD59 deficiency is the abnormality that is responsible for the hemolysis and thrombosis classic of PNH.

Venous thrombosis in PNH

The most feared complication of PNH is venous thrombosis, which has a predilection for the intra-abdominal and cerebral veins. In two historical series of patients, approximately 50% experienced venous thrombosis at some time during their disease and a third of patients died as a result of thrombosis.[7,8] The risk of thrombosis is greater in patients from Europe and the United States than in patients from the Far East.[9] A possible explanation is that aplastic anemia (AA) is more prevalent in the Far East and therefore patients are more likely to have PNH/AA rather than hemolytic disease. Alternatively patients from different ethnic groups may have different additional inherited prothrombotic traits—however, no correlation between the inherited thrombophilia and thrombosis in PNH has been demonstrated.[10] The cause of the thrombotic tendency in PNH is not entirely clear. The overwhelming evidence implicates the GPI-deficient platelets, which are more easily activated by complement than normal platelets. Thus PNH platelets, which comprise the vast majority of platelets in almost all hemolytic patients, similar to the proportion of PNH neutrophils, undergo microvesiculation at far lower concentrations of activated complement leading to greater prothrombinase activity and to thrombus formation.[11] Alternative mechanisms have been suggested, such as deficiency of urokinase plasminogen activator receptor from PNH neutrophils or directly due to intravascular hemolysis, but the platelet abnormalities appear to be the most important factor.[11,12]

Nitric Oxide and the Symptoms of PNH

It is likely that the symptoms of PNH during a paroxysm, such as esophageal spasm and abdominal pain, are caused by smooth muscle dysfunction due to disturbances in the metabolism of nitric oxide (NO). These symptoms are identical to those observed when free hemoglobin was given to normal individuals during the early attempts to produce artificial blood and can be induced by the inhibition of NO synthetase in normal individuals. Haptoglobin efficiently removes free hemoglobin and is necessary because the function of hemoglobin depends upon its correct compartmentalization in red cells. Free hemoglobin, as is seen in hemolytic PNH, is an extremely efficient scavenger of NO with

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10^6 times greater affinity of the heme moiety for NO than that for oxygen. Thus intravascular hemolysis absorbs NO, disturbs smooth muscle function and causes the symptoms seen during a paroxysm.

Conventional Therapeutic Strategies in PNH

The principal conventional therapy for PNH is supportive care with transfusions as required and the treatment of complications, such as thrombosis, when they occur. The only curative strategy is allogeneic stem cell transplantation but this carries a considerable risk of mortality from the reported series[13] and, in view of the fact that a proportion of patients will eventually experience a spontaneous remission of PNH (occurs in approximately 15% of hemolytic patients)[7] and with the advent of potentially effective novel therapies, this should only be considered in selected cases, such as those with a syngeneic donor or with associated bone marrow failure. In these patients the indications for transplantation are similar to those for AA. Patients with cytopenias due to associated AA will often respond to immunosuppressive therapy with antilymphocyte globulin and/or cyclosporine. The occurrence of venous thrombosis affecting a major vessel is a life-threatening occurrence and the advent of reduced-intensity conditioning allogeneic stem cell transplantation, which carries a lower risk than conventional bone marrow transplantation (BMT), has led to this approach being used in some patients with life-threatening thromboses.

Diagnosis of PNH

Flow cytometry

The gold standard diagnostic test for PNH is the analysis of GPI-linked molecules on the surface of hematopoietic cells by flow cytometry (see **Figure 1**).[14] An unusual feature of flow cytometry for PNH diagnosis is that a positive test is defined by an absence of antigens rather than the aberrant expression of antigens as seen in most other flow cytometric applications. However in almost all PNH patients there is at least a small residuum of normal cells, which acts as an internal control to demonstrate that the staining and gating strategy are correct. The minimum requirement to diagnose PNH is the demonstration that a proportion of red cells are deficient in at least two different GPI-linked molecules. The proportion of GPI-linked deficient granulocytes is not affected by either hemolysis or transfusions and therefore gives the best estimate of the actual proportion of PNH hematopoiesis. In addition to establishing the diagnosis of PNH, flow cytometry also provides information regarding the severity of deficiency of the antigens from PNH red cells. PNH Type III red cells

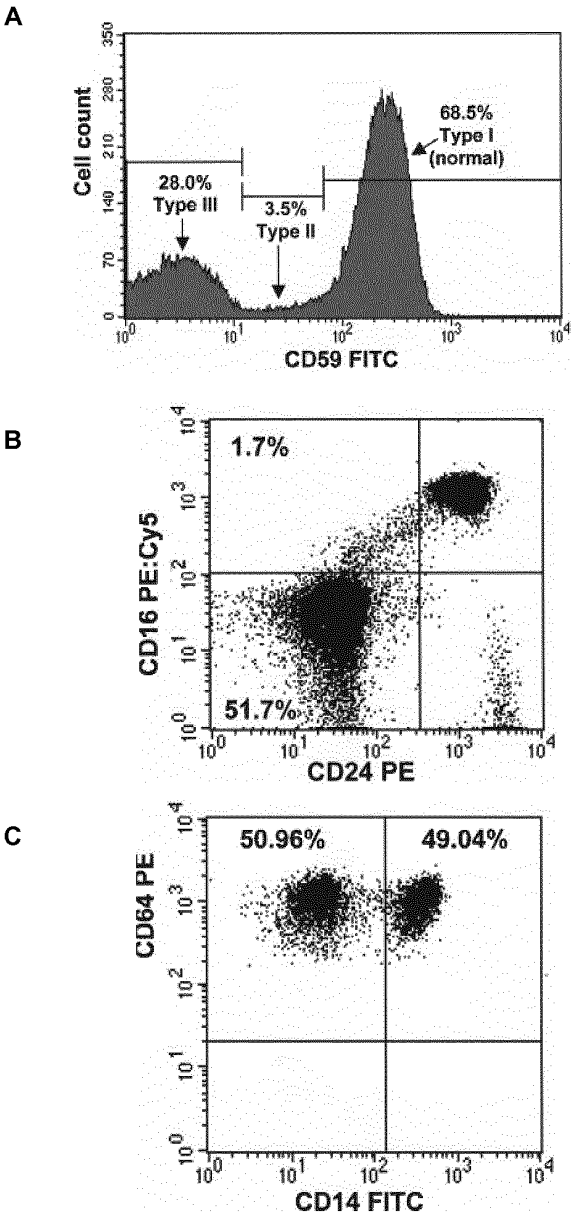


Figure 1. Flow cytometry to diagnose paroxysmal nocturnal hemoglobinuria (PNH) and to quantitate the clone.

A. Demonstrates a population comprising 28% of the red cells that are completely deficient in CD59 (and other glycosylphosphatidylinositol (GPI)–linked antigens) and a second abnormal population of partially deficient red cells comprising 3.5% of the total.

B. Demonstrates the lack of more than one GPI-linked antigen (CD16 and CD24) from the same patient's granulocytes.

C. Demonstrates that the proportion of PNH monocytes usually closely corresponds to the granulocyte analysis. (Courtesy of Dr. S.J. Richards)

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are completely deficient in GPI-linked molecules and are 15 to 25 times more sensitive to activated complement (compared to normal) whereas PNH Type II red cells have a partial deficiency (PNH Type II cells) and are only 3 to 5 times as sensitive. The size of the PNH clone and type of PNH cells are important determinants of the clinical presentation. For example, patients with hemolytic anemia and macroscopic hemoglobinuria will almost always have over 10% PNH Type III red cells and a majority, often over 95%, of PNH neutrophils. The risk of thrombosis is directly related to the proportion of PNH neutrophils, and this information has been used to stratify patients when deciding who should receive warfarin as primary prophylaxis against venous thrombosis.

Tests of complement sensitivity

Other tests, such as the demonstration that PNH red cells have an increased sensitivity to complement (the Ham test), are less sensitive than flow cytometry. These may be useful screening tests but can no longer be relied on to establish the diagnosis. The diagnosis should always be confirmed by flow cytometry. An alternative diagnostic approach is to utilize the toxin Aerolysin, which is produced by the bacteria *Aeromonas hydrophila* and binds to the GPI structure.[15] Aerolysin has been fluorescently labeled (FLAER), and therefore any nucleated cell that expresses GPI-anchored antigens is positive, whereas the GPI-deficient cells remain negative. The FLAER test is currently under investigation as a diagnostic test for PNH.

PNH Pathophysiology: Relative Growth Advantage of the PNH Clone

PNH is a unique disorder in which an abnormal clone or small number of clones expand to replace almost the entire hematopoietic stem cell pool, but these clones do not have any "malignant" tendency in that they appear to be regulated in a normal manner with no tendency to metastasize beyond the normal hematopoietic compartment. Also GPI-deficient cells with *pig-a* mutations occur very frequently at low levels in normal individuals but do not expand in competition with the normal hematopoietic cells. Dacie first proposed that in order to develop PNH two things were required: first, the occurrence of a GPI-deficient clone arising in a multipotent hematopoietic stem cell; second, a second event that encourages the expansion of the PNH clone over the residual normal hematopoiesis—a relative growth advantage for the PNH cells.[16] The clue to this second event is the close relationship between aplastic anemia and PNH. It appears that normal hematopoiesis is suppressed by the immune system, presumably either directly or indirectly through one or more GPI-linked antigens, and therefore this attack spares the GPI-deficient PNH clone. Thus, in an environment where there is intense pressure for hematopoiesis (aplastic anemia), the PNH clone is driven to produce mature hematopoietic cells and expands to fill the void left by the aplastic process. The exact mechanism for the relative growth advantage of PNH cells remains unclear but is the subject of considerable scientific activity.

Novel Therapeutic Strategies in PNH

Prevention and treatment of thrombosis

The first thrombosis in a patient with PNH heralds a significant deterioration in the patient's health and a worsening prognosis. Reports of tissue plasminogen activator (tPA) for intra-abdominal venous thrombosis in PNH show that the thrombus can be cleared effectively in a proportion of patients.[17] The standard of care after established venous thrombosis in PNH is life-long full anticoagulation. In view of the high risk of thrombosis and the fact that patients have decreased quality of life following the first thrombosis, selected patients could be considered for warfarin as primary prophylaxis prior to the thrombosis. Hall et al[18] recently reported a retrospective analysis comparing 39 patients at high risk of venous thrombosis treated with warfarin as primary prophylaxis to 56 patients with similar sized clones who were not treated with warfarin. The incidence of thrombosis at 10 years in the group of patients not on warfarin was 36.5%, which was statistically significantly higher than the warfarin group in whom no patients had a thrombosis. However, 2 patients in the warfarin group had major hemorrhages, with 1 dying as a direct result of an intracranial bleed. Despite this being a retrospective analysis and the fact that the patients were not randomized the results suggest that primary prophylaxis may have a role (**Figures 2** and **3**). The data are compelling, particularly in view of the fact that because the patients in the warfarin group were selected for anticoagulation they would be expected to have at least as high a risk of thrombosis as the non-warfarin group. PNH patients with neutrophil clone sizes of over 50%, platelet counts above 100×10^9/L and no other contraindication for warfarin therapy should be considered for primary prophylaxis. There are no studies of antiplatelet drugs, such as aspirin or clopidogrel, in PNH.

Inhibition of the complement cascade

Complement is the principal effector of the innate immune system. Complement activation is a complex cascade leading to the production of anaphylatoxins,

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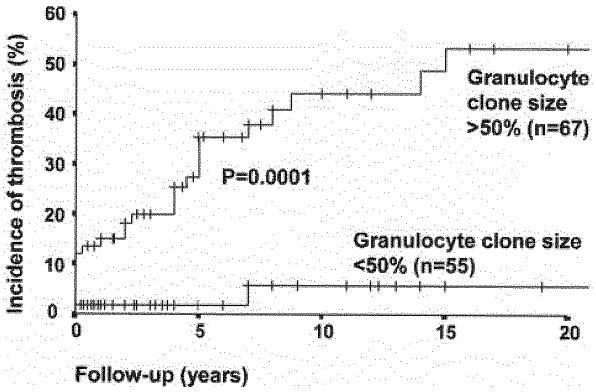


Figure 2. Effect of glycosylphosphatidylinositol (GPI)-deficient granulocyte clone size on incidence of venous thrombosis (primary prophylaxis patients excluded).

When primary prophylaxis patients are excluded, the 10 year cumulative incidence rate of thrombosis in patients with PNH granulocyte clone size of > 50% is 44%, compared with a thrombosis rate of 5.8% in those with clone size of < 50% (*P* < 0.01*).

* *P* value calculated with use of the log-rank test; Hall et al. Blood. 2003;102:3587-3591.

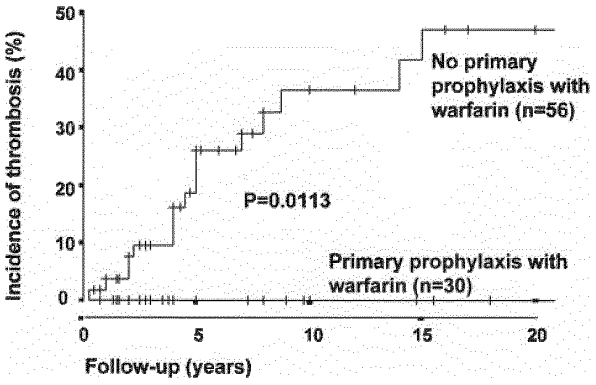


Figure 3. Effect of warfarin prophylaxis on venous thrombosis in patients with paroxysmal nocturnal hemoglobinuria (PNH) granulocyte clone sizes of > 50% (patients presenting with thrombosis excluded).

The 10-year cumulative incidence rate of venous thrombosis in patients with PNH granulocyte clones of > 50%, not presenting with thrombosis and not taking warfarin is 36.5%. In comparison, the current thrombosis rate is 0% in patients taking primary prophylaxis (*P* = 0.01*).

Thirty-two of the 39 patients on primary prophylaxis had granulocyte clone sizes > 50% and could therefore be included in this analysis. A further 2 of these patients were excluded because, having stopped warfarin (one through personal choice and one because of warfarin-associated hemorrhage), they went on to suffer venous thrombosis.

Time 0 was the time of presentation with PNH.

* *P* value calculated with use of the log-rank test; Hall et al. Blood. 2003;102:3587-3591.

chemotaxins and the membrane attack complex (MAC). Three pathways activate the cascade (see **Figure 4**): the classical (initiated by antigen/antibody complex), alternative (initiated by microbial membranes or immune complexes), and lectin pathways. These pathways all converge to cleave C5 into C5a, a potent anaphylatoxin, and C5b. C5b is the initial molecule of terminal complement and binds C6, then C7, and then C8. C5b-8 forms the scaffold for C9 molecules, which bind to each other to form the MAC. The MAC forms a pore in the cell membrane, which results in cell lysis. CD59 prevents the incorporation of C9 onto C5b-8. A remarkable feature of the complement cascade is that the MAC appears to be largely redundant in normal adults. Individuals with inherited deficiency of any of the complement molecules prior to C5 are vulnerable to both pyogenic organisms and to autoimmune disorders. In contrast, deficiency of any of the molecules after C5 remarkably has little phenotype. The only apparent se-

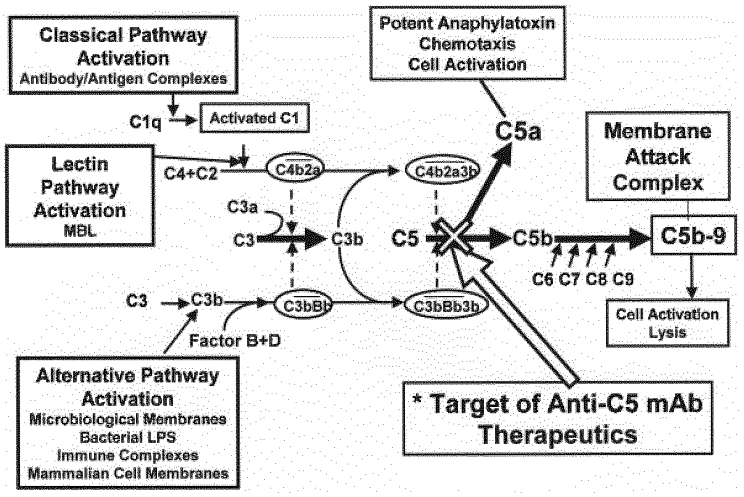


Figure 4. The complement cascade and eculizumab.

The complement cascade culminating in the production of the membrane attack complex that results in the lysis of the cell. The cleavage of C5 is the pivotal point of the pathway. Inherited deficiencies prior to C5 result in recurrent pyogenic infections and autoimmune disorders, whereas deficiencies after C5 have remarkably little effect except for an increased risk of infection by encapsulated organisms. The site of blockage of eculizumab is demonstrated.

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quela is an increased risk of infection by encapsulated organisms, namely *Haemophilus influenzae, Neisseria meningitides* and *N gonorrhoea*. In the case of *N meningitides,* although the deficient individuals have an increased risk of suffering infection they also have a significantly lower mortality from infection when compared to complement-replete individuals. C5 is a good therapeutic target, because blockade here would not only prevent the creation of MAC but would also prevent the release of the potent anaphylatoxin C5a. Eculizumab is a humanized chimeric antibody against C5 with a completely nonfunctional Fc domain. Eculizumab has a high affinity for C5 and thus when bound remains so until the complex is removed from the circulation. Recently a pilot study of eculizumab in 11 patients with transfusion-dependent PNH was reported.[19] The antibody was given at a dose of 600 mg every week for 4 weeks and then at a dose of 900 mg every other week. The drug is given by intravenous infusion over 30 minutes and was extremely well tolerated. The parameters of intravascular hemolysis, namely lactate dehydrogenase (LDH) and aspartate transaminase (AST), immediately fell to normal or near normal levels. The mean LDH prior to eculizumab was 3111 ± 598 IU/L (normal range 150–480) and this fell by the first week to 594 ± 32 IU/L. There was a significant improvement in the quality of life when comparing before the start of eculizumab and at week 12. There was also a significant reduction in mean transfusion requirement for the whole group from 2.1 units per month to 0.6 units per month. The benefit was most evident in patients with normal or near normal platelet counts and therefore no evidence of co-existent clinically significant marrow failure. There was also a complete cessation of hemoglobinuria immediately after commencing eculizumab. The proportion of PNH red cells in patients not on eculizumab is usually significantly lower than the proportion of PNH neutrophils because of the effect of transfusions and also the selective hemolysis of PNH red cells compared to their normal counterparts. The reduction or cessation of transfusions and control of hemolysis in patients on eculizumab leads to an increase in the proportion of PNH red cells toward the level of the PNH neutrophils. The median proportion of PNH type III red cells increased from 36.7% ± 5.9% prior to eculizumab to 59.2% ± 8.2% by week 12 of therapy. This raises the concern that stopping eculizumab for any reason might render the patient susceptible to a massive hemolytic attack and profound anemia. Two of the patients on eculizumab have had a breakthrough from complement blockade immediately prior to the next dose of eculizumab with a return of hemoglobinuria. Although both suffered hemolysis, the severity of the attack was not too extreme and in both the complement was reblocked immediately after the next dose of eculizumab and remained so on a slightly more frequent dosing regimen. Although eculizumab appears to almost completely stop the intravascular hemolysis, the patients' hemoglobin does not return to normal (it usually plateaus between 10 and 12 g/dL), the bilirubin and reticulocytes remain elevated and the haptoglobin initially becomes detectable but reverts to undetectable within a few weeks of the start of therapy in almost all patients. Thus a continuing low level of well-compensated extravascular hemolysis persists, suggesting that the hemolysis in PNH is not only due to terminal complement activity but that there is also a component of extravascular hemolysis due to complement activity prior to C5. Eculizumab appears to be a highly promising therapy for PNH and is now the subject of a Phase III randomized clinical trial.

Replacement of complement regulatory proteins on PNH cells

An alternative therapeutic strategy in PNH could be to replace CD59, the deficient complement regulatory protein. In the first instance a gene therapeutic[20] approach might appear attractive. However, introducing the *pig-a* gene into the PNH hematopoietic stem cell is far from trivial and would simply render the "corrected" cell as a target for the aplastic process that was the reason for the proliferation of the PNH clone. An alternative strategy would be to simply replace CD59 on the PNH red cell surface. It is impractical to extract GPI-linked CD59 from cell membranes as it is extremely tightly bound and does not re-attach well to cells in the presence of albumin. CD59 is functional when bound to the cell by alternative mechanisms, such as a transmembrane linkage, but again this approach is impractical.[21] Sah et al[22] have recently reported the use of an alternative artificial glycolipid anchor (Prodaptin) to anchor CD59 into the cell. Prodaptin-CD59 has been shown to effectively coat PNH cells in vitro and murine cells in vivo and to restore the cell's resistance to complement. This approach is attractive because the complement system is not blocked and the increased risk of *N meningitides,* which is seen in complement deficient individuals, is no longer an issue.

Global PNH Registry

In view of the infrequency of PNH it is extremely difficult to perform large or randomized trials of therapeutic interventions. In addition, the advent of complement inhibitors, such as eculizumab, or the routine use of prophylactic anticoagulation promises to alter the natural history of PNH. In fact, the approval process of

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eculizumab as a therapeutic agent will be on the basis of relatively small and short clinical trials. Thus few patients are likely to have received more than 3 years of eculizumab. It is possible that any complications of such a novel agent might take several years to develop. These factors have led to the recent development of a Global Registry for PNH. It is hoped that a large number of PNH patients will be registered on the Global PNH Registry in order that a more thorough insight into the effect of these changes on the natural history of PNH can be established (www.PNHregistry.org).

Conclusion

The understanding of the biology and natural history of PNH has altered dramatically over the last 10 years. This has facilitated major steps forward in our ability to diagnose the disorder and in our therapeutic strategies for PNH. There is now initial evidence that we may be able to prevent the most feared complication of PNH, namely thrombosis, with the use of primary prophylaxis with anticoagulants for selected patients. The recent description of a small pilot study of the complement inhibitor eculizumab offers the first promise that we may have a "targeted" therapy capable of controlling the hemolysis of PNH. Other novel approaches to therapy in PNH are also being explored. The development of the Global PNH Registry offers the potential to further our understanding and therefore therapy of PNH.

II. Fcγ Receptor Structure/Function and Role in Immune Complex–Mediated Autoimmune Disease

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Antigen recognition by cells of the immune system occurs via several mechanisms. One important family of receptors involved in the recognition of immunoglobulin (Ig)-coated particles and complexes are Fc receptors. Fc receptors recognize the Fc portion of Ig and are accordingly grouped into subfamilies. They are named depending upon which class of Ig they bind. The major Fc receptors are Fcγ receptors that bind IgG, FcαR that bind IgA, and FcεR that bind IgE.[1] Fc receptors are responsible for such functions as endocytosis, phagocytosis, granule release, reactive mediator release and cell activation/cytotoxicity. Fc receptors are found on specific cell types corresponding to their ability to recognize Ig. As such, Fcγ receptors are found primarily on neutrophils, macrophages and monocytes where they can detect and phagocytose IgG-coated particles, and on B lymphocytes.

Fc Receptor Subtypes and Expression Patterns

Fc receptors can be divided into two groups based on their signaling capability and structure. The first and most common type of Fc receptor is a multichain heterocomplex composed of a ligand-binding α-chain and one or more signal-transducing γ -chains. The second type of Fc receptor is a single-chain transmembrane receptor containing a signal-generating motif(s) in the cytoplasmic domain and, thus, not requiring another signal-transducing subunit.

Receptors for a specific isotype of IgG can vary in structure and can differ in ligand affinity and signaling ability.[1] Fcγ receptors are categorized into three classes: Fcγ RI, Fcγ RII, and Fcγ RIII (**Figure 5**; see Color Figures, page 514). Fcγ RI is a high affinity receptor that can be subdivided into three groups (A, B and C) that are encoded by three different genes. Fcγ RI is expressed on monocytes, macrophages, neutrophils and some dendritic cells. The receptor can be upregulated upon stimulation with interferon (IFN)-γ , tumor necrosis factor (TNF)-α or granulocyte colony-stimulating factor (G-CSF).[2,3] Similar to Fcγ RI, Fcγ RII has been divided into three families encoded by three genes, named A, B and C, which are of relatively low avidity for monomeric IgG but of high avidity for complexed IgG. Fcγ RII is expressed on various cell types and contributes to cell function based on the subclass of Fcγ RII. Fcγ RIIA is expressed on neutrophils, monocytes, macrophages, natural killer cells and platelets. Fcγ RIIB is expressed in two forms following alternative splicing, forming the Fcγ RIIB1 and Fcγ RIIB2 isoforms, and is limited in expression to B cells, neutrophils, macrophages and monocytes. Fcγ RIIC is expressed on human natural killer cells. Fcγ RIII is divided into two families, the transmembrane form Fcγ RIIIa and the GPI-linked Fcγ RIIIB found on neutrophils and natural killer cells. Fcγ RIIIA is found on monocytes and macrophages, and has also been shown to be expressed and upregulated on eosinophils.[4]

Crystal Structure/Affinity for IgG

Both recent immunotherapy using IgG antibodies as well as certain antibody-mediated autoimmune diseases utilize Fcγ receptors for the clearance of IgG-coated cells from circulation. While this may be beneficial in immunotherapy, it is detrimental in autoimmune disease. Understanding the interaction between the Fc domain of IgG and the Fcγ receptors is necessary in order to develop better therapy. There are two extracellular

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immunoglobulin-like domains for Fcγ RII and Fcγ RIII, while Fcγ RI has three immunoglobulin-like domains. The third domain of Fcγ RI has been attributed to the high affinity binding of IgG by this receptor; however, since the extracellular region of Fcγ RI has not been crystallized this has not been confirmed experimentally. The crystal structures of the extracellular region of Fcγ RIIA, Fcγ RIIB, Fcγ RIIIA, and Fcγ RIIIB have been determined.[5] The two immunoglobulin-like domains of these receptors, D1 (membrane distal) and D2 (membrane proximal), are bent at an approximately 50–70° angle relative to each other depending on the algorithm used. All Fcγ receptors are structurally very homologous, with their domains oriented in a steep angle to each other. While most studies have shown Fcγ receptors existing in the membrane as a monomer, some conflicting reports have suggested that Fcγ RIIa forms a homodimer, with a 2:1 stoichiometry of receptor to IgG.[6] However, the crystallization of Fcγ RIIIa in complex with the Fc region of IgG1 suggests a 1:1 stoichiometry of receptor to IgG.[7] It is believed that the other Fcγ receptors display similar binding stoichiometry due to their high degree of homology.[8] Also, as a dimer is able to crosslink Fc receptors to induce signaling, it may not be efficient for cells to be able to induce signaling by binding a single IgG molecule. However, the conformation of both bound and unbound Fcγ RIIa remains a matter of debate.

The binding sites of the Fcγ receptors for IgG are fairly well established. From the crystallization studies as well as some mutational studies, the D2, or membrane proximal, domain of the Fcγ receptor is important for the binding of IgG. The binding of an IgG by the receptor induces approximately a 10° shift in the angle between the D1 and D2 domains.[7] The Fcγ receptor binds asymmetrically to an IgG molecule in the lower hinge region of the Cγ 2 domains of an IgG heavy chain. The asymmetrical binding may result from a conformational change in one of the Cγ 2 domains of IgG in relation to the other. A factor that could affect the IgG binding ability of the Fcγ receptor is the glycosylation state of the receptor and IgG. One example of this is found in the crystal structure of Fcγ RIIIa/IgG-Fc, whereby the glycosylation of the IgG1 at 297N was not involved in the binding site. However, two groups have shown that glycosylation of an IgG may be involved with the formation of a stable Cγ 2-Cγ 2 interaction of the two heavy chains of IgG1. When the carbohydrates are removed, there is decreased binding of IgG1 to both Fcγ RIII and Fcγ RIIb (**Table 1**).[9,10] The glycosylation of Fcγ receptors can also affect binding of IgG. Expression of Fcγ RIIIa on different cell types leads to different glycosylation patterns. The differences in the ability to bind monomeric IgG by Fcγ RIIIa have been attributed to the cell type specific glycosylation of the receptor.[11] Also, one particular glycosylation site in the D2 domain of Fcγ RIIIa and Fcγ RIIIb, 163N, is located in the IgG-binding site. Receptors lacking glycosylation at this amino acid have an increased affinity for IgG.[12] Thus, the glycosylation state of the receptor, which may vary in cases of inflammation, could affect the binding of IgG.

Another example of affinity modulation is through polymorphism. As such, a polymorphism in Fcγ RIIa (131R or 131H) affects the binding affinity of Fcγ RIIa for human IgG2; Fcγ RIIa (131H) binds IgG2 but Fcγ RIIa (131R) does not.[13] Results of a recent meta-analysis show that the Fcγ RIIa polymorphism is important in genetic susceptibility for systemic lupus erythematosus (SLE).[14] There is also a polymorphism in Fcγ RIIIa (158V or 158F) that affects binding to human IgG1 and human IgG3. NK cells from patients with Fcγ RIIIa (158F) are unable to bind IgG3 and bind IgG1 with lower affinity, whereas NK cells from patients with Fcγ RIIIa (158V) are able to do so.[15] In addition, a triallelic polymorphism in Fcγ RIIIa (48 H, R, or L) was originally reported to affect the binding affinity for human IgG. Subsequently, this difference in binding affinity was determined to be due to the polymorphism at amino acid 158.[16] This is consistent with structural studies as 48 H/R/L is located in the D1 immunoglobulin-like domain whereas 158 V/F is located in the D2, IgG-binding domain. Finally, in Fcγ RIIIb, the GPI-linked Fcγ receptor expressed on neutrophils, there are two alleles (NA1 and NA2). These two alleles have minimal differences in binding affinity for IgG, but patients homozygous for the NA1 allele have a higher level of phagocytosis of IgG-coated particles.[17]

Table 1. Relative affinities of IgG subclasses for various Fcγ receptors.

	Fcγ RI	Fcγ RIIa	Fcγ RIIb	Fcγ RIII
Molecular Mass	70 kDa	40 kDa	40 kDa	50-80 kDa
IgG Subclass Specificity	1 ≥ 3 > 4 > 2	3 > 1 > 2 > 4	3 ≥ 1 > 4 > 2	1 = 3 > 2 = 4
IgG Affinity	10^{-7}–10^{-9} M	> 10^{-7} M	> 10^{-7} M	> 2 × 10^{-7} M

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Signal Transduction from Fcγ Receptors

Given that most Fcγ receptors are transmembrane proteins, the first step of receptor activation and subsequent phagocytosis is binding of IgG containing complexes to the receptor extracellular domain. Binding and phagocytosis by human Fcγ receptors has been observed both in normal human leukocytes and in model systems such as transfected COS-1 cells.[18] Initial Fcγ receptor activation takes place upon ligand binding to the extracellular domain. Since each Fcγ receptor has a structurally distinct extracellular domain, the receptor binds to IgG with varying affinity, as shown in **Table 1**.

Fcγ receptors traditionally signal through an immunoreceptor tyrosine–based activation motif (ITAM) or through inhibitory residues found in an immunoreceptor tyrosine–based inhibitory motif (ITIM).[19,20] ITIMs are composed of an I/VxYxxL/I sequence that recruits tyrosine phosphatases to the signaling complex. Therefore, the presence of ITIM-bearing receptors imposes a negative effect. As such, the presence of Fcγ RIIB, an ITIM-bearing receptor, inhibits phagocytic signaling mediated by activating Fcγ receptors.[21]

The classic ITAM motif consists of two YxxL sequences separated by 7 amino acids.[22] Fcγ receptor ITAM sequences in the Fc receptor associated γ -chain abide by this structure. However, the cytoplasmic domain of Fcγ RIIa contains an ITAM-like domain. This ITAM-like domain contains the two YxxL motifs but a spacer sequence containing 12 amino acids instead of the usual 7.[20] ITAM tyrosine residues have been shown to be crucial for mediating the phagocytic response. When either of the ITAM tyrosines are mutated to phenylalanine, phagocytosis is inhibited by ~70%–80%. However, if both tyrosine residues are mutated phagocytosis is abolished.[23-25]

It has been proposed that ITAM tyrosines are phosphorylated by Src family kinases after crosslinking. Several members of the Src family have been shown to associate with specific Fcγ receptors. However, which Src kinase is responsible for phosphorylation of a specific Fc receptor is not established. Studies have been somewhat inconclusive in elucidating which kinase is responsible for phagocytic signaling through each Fcγ receptor. An example of these observations can be found in knockout experiments where phagocytosis is not abolished in Hck, Lyn, or Fgr single knockouts.[26] In addition, in triple knockout mice, phagocytosis by macrophages and neutrophils is still partially intact, suggesting other kinases may play a redundant role in phagocytosis.[27]

Phosphorylation of ITAM tyrosines creates Src homology 2 (SH2)-binding sites required for signal transduction involving other members of the tyrosine kinase family.[28-30] Most importantly, Syk tyrosine kinase, which contains two SH2-binding sites, is recruited to phosphorylated ITAM residues (**Figure 6**). Fcγ R phagocytosis is dependent upon Syk signaling, and has been observed to be enhanced upon the overexpression of Syk in model systems.[31] In addition, when Syk kinase expression is inhibited with antisense oligonucleotides both in vitro and in vivo, phagocytosis and inflammation are abolished.[32,33]

SH2-containing proteins are important in signaling complexes. For example, recent studies have emphasized the role of adaptor proteins in phagocytic signaling. Adaptor molecules such as SLP-76, LAT, Cbl and others have the ability to recruit SH2-containing proteins to sig-

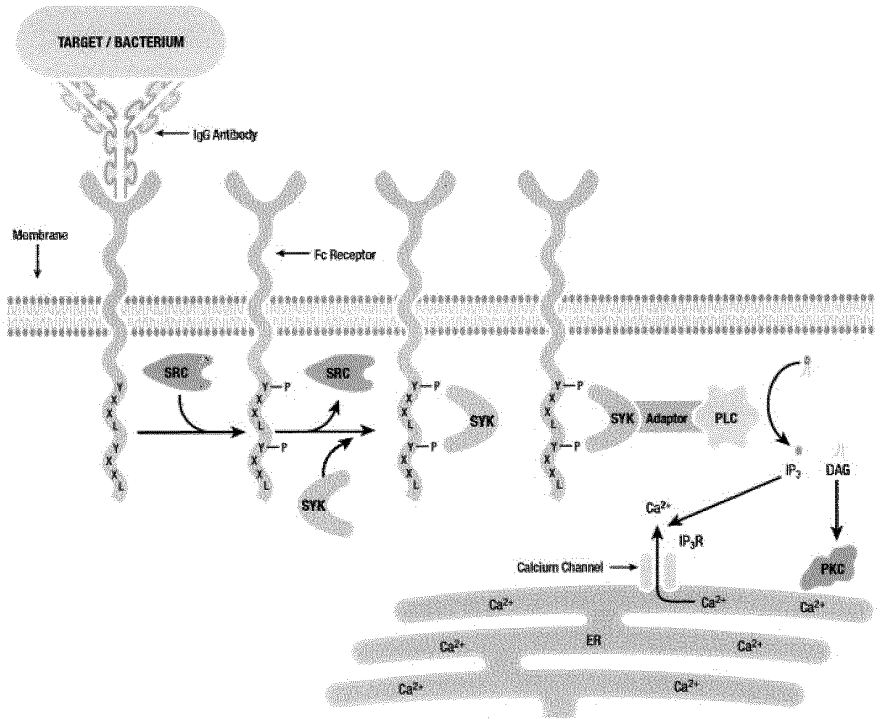


Figure 6. Proximal signal transduction by activating Fcγ receptors.

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naling complexes, notably in lipid rafts (**Figure 6**). These adaptor proteins play a significant role in recruiting such secondary signaling molecules as phospholipase C (PLC), Grb2, Shc and others. The ability for adaptor molecules to recruit proteins to the site of signal propagation by Fcγ receptors is important for efficiently triggering the downstream signaling leading to target internalization and mediator release.

Fcγ Receptor–Associated Diseases

As described above, the isotype of IgG determines the class of Fcγ receptor ligated and the nature of the signal propagated. Of particular interest are the myriad of diseases based upon IgG-Fcγ receptor interaction leading to inflammation, phagocytosis and endocytosis. Two particular diseases mediated by Fcγ receptors are immune thrombocytopenic purpura (ITP) and autoimmune hemolytic anemia (AIHA). Both of these diseases are caused by production of self-reactive antibodies against either platelet antigens or erythrocyte antigens. ITP is mediated by production of IgG against one or more antigens exposed on platelets. Specifically, antibodies reactive to platelet GPIIb-IIIa, GPIb-IX, GPIb and GPIIIa among others have been shown to be potentiators of ITP.[34] AIHA has been shown to be due in large part to self-reactive antibodies against erythrocyte Band 3, an ion transporter found in erythrocyte membranes also shown to be involved in erythrocyte senescence. Both of these diseases are mediated by Fcγ receptors found on phagocytes (macrophages) as part of the reticuloendothelial system (RES) in the spleen and liver and may lead to thrombocytopenia or anemia, respectively.[34]

Various observations by multiple laboratories have determined the role of Fcγ receptors in these diseases most conclusively in murine models lacking the common γ -chain (γ KO). Experimentally induced AIHA and ITP are markedly decreased in mice lacking the γ -chain.[35] Additionally, administration of monoclonal antibody 2.4G2, which binds to and blocks mouse Fcγ RII and Fcγ RIII, allows a rapid recovery after induction of AIHA or ITP. Similarily, administration of GM-CSF has been shown by various groups to accelerate AIHA potentially due to upregulation of Fcγ RI.[36] These reports show that altering the balance of stimulatory to inhibitory Fcγ receptors has a marked effect on disease progression and susceptibility. As such, viral infection has been shown to stimulate or increase susceptibility to both AIHA and ITP. However, the mechanism(s) by which viral infection does so has yet to be fully elucidated. Potential scenarios of viral infection that could lead to this outcome include stimulated production of IFNγ during viral infection that causes upregulation of Fcγ RI. Alternatively, viral infection may cause a change in the expression pattern of Fcγ receptors due to transcriptional activation or other mechanisms. For example, childhood ITP has been described after infection with varicella zoster virus (VZV), causing molecular mimicry of antibodies against VZV to crossreact with platelet antigens, thus causing ITP.[37] ITP in some patients has been shown to be due to production of predominantly self-reactive IgG1.[38]

Therapy for ITP or AIHA

Treatment for ITP and AIHA has recently been reviewed elsewhere.[39,40] Many of the therapies used involve altering Fcγ receptor expression or phagocytosis. One of the first treatments is the administration of glucocorticoids. Prednisone and dexamethasone have been shown to downregulate Fcγ receptor expression, thus potentially decreasing the phagocytosis of IgG-coated particles. Other treatments involve administration of anti-D IgG to Rh-D positive patients. The rationale behind this treatment is that the IgG will coat erythrocytes with subsequent binding by Fcγ receptors. Binding of the IgG-coated red blood cells will prevent the binding and phagocytosis of the IgG-coated platelets. However, self-limited hemolytic anemia is to be expected after anti-D treatment.

High-dose intravenous immunoglobulin (IVIG) is also used as a treatment for both ITP and AIHA. However, the mechanism by which this inhibits the clearance of the IgG-coated platelets or IgG-coated red blood cells is still unknown. It is believed to work through a number of different mechanisms, which may include anti-idiotypic antibodies and decreased autoantibody production,[41] and, of interest here, its effects on Fcγ receptor function. One principal theory for a mechanism of IVIG's function is through a blockade of Fcγ receptors. At high local concentrations of IgG, the IVIG may either bind to the low-affinity Fcγ receptors, preventing the binding of IgG-coated cells, or outcompete binding of the high avidity immune complexes. Also, the possibility of IgG dimers existing in IVIG preparations would even further enhance this blockade.[42] Another possibility involves the inhibitory Fcγ RIIb, which is able to inhibit phagocytosis mediated by activating Fcγ receptors.[21] Injection of IVIG into a mouse model of ITP increased circulating platelet counts.[43] Also in this model was an increased percentage of non B cell splenocytes expressing Fcγ RIIb. Increased expression of this receptor relative to the expression of activating receptors could decrease the phagocytosis of IgG-coated platelets. The mechanism for the IVIG-mediated increase in Fcγ receptor–expressing cells is still unknown.

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Summary
Fcγ receptors are integral for the removal of IgG-coated cells from the circulation. In autoimmune diseases such as autoimmune hemolytic anemia and immune thrombocytopenia the removal of the IgG-coated erythrocytes or IgG-coated platelets is detrimental to a patient. Much is known regarding the structure and signaling properties of the Fcγ receptor family. As the two autoimmune diseases mentioned involve phagocytosis of IgG-coated cells, interference at either the Fcγ receptor binding of these complexes or interference of the phagocytosis process could possibly improve on the current treatments available for these disorders. Understanding the crystal structure of the Fcγ receptors may lead to the development of molecules that inhibit the binding to an IgG-coated cell. Small molecules might inhibit Fcγ receptor signaling at many different steps leading to phagocytosis. Finally, treatment to decrease expression of the activating Fcγ receptor or increasing expression of the inhibitory Fcγ receptor may also prove to be an effective therapy.

III. Cold-Induced Immune Hemolytic Anemia

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The antibodies that cause immune hemolytic anemia can be classified by isotype (IgG, IgM, or IgA) and by the temperature at which they react maximally with the antigen on the red cell: warm reacting if that temperature is 37°, cold reacting if that temperature is less. Much of the pathophysiology and many of the clinical manifestations are determined by these characteristics. For the most part, the isotype and the temperature of reaction coincide: most frequently, cold-reacting antibodies are IgM and warm-reacting antibodies are IgG, but important exceptions exist. Two clinical entities due to cold-reacting antibodies are defined by differences in isotype: cold agglutinin disease is caused by IgM antibodies and paroxysmal cold hemoglobinuria by IgG antibodies.

The isotype is important because the isotypic characteristics of the antibody direct the mechanisms of destruction. As described in Section II, phagocytic cells have receptors specific for IgG molecules of specific subtypes (IgG1, IgG2, and IgG3) but not for IgM molecules; therefore, Fc-receptor–induced destructive processes apply only when IgG molecules are attached to the red cell in the presence of the phagocytic cells. On the other hand, IgM antibodies fix complement much more readily than IgG molecules because the two necessary attachment sites for C1q are present on a single molecule; therefore, complement plays a primary role in the destruction of the red cells by these antibodies.[1,2]

Why Are Cold-Reacting Antibodies Cold Reacting?

The interaction of antigen and antibody is reversible and the degree of attachment depends upon the forces interacting between the two. For all cold-reacting antibodies, the antigen with which they react is polysaccharide or the polysaccharide parts of glycoproteins. In the case of cold agglutlinins, the antigen is one of the following: the straight-chain paragloboside basic to the expression of the ABH antigens (the i antigen), a branched version of that molecule (the I antigen), polysaccharides resident on glycoproteins (Pr antigens), and rare sialylated polysaccharides. Since the branching enzyme responsible for the I antigen is not activated until after birth, cord cells (and the cells of individuals genetically lacking the branching enzyme) express the i antigen whereas the red cells of adult express the I antigen.

The interaction of antibody and polysaccharide is dependent upon weak forces that are easily disrupted by molecular activity that occurs at higher temperatures. The cold agglutinins of anti-I or anti-i specificity are strikingly similar to one another in the structure of the antigen binding site. These antibodies all react with a monoclonal antibody that identifies the product of the VH4-34 (VH4-21) gene segment.[3] Other antibodies (monoclonal anti-Rh system antibodies, etc.) have been shown to use this same gene segment for the variable portion of the heavy chain and many of them also have cold agglutinin activity against the I/i antigens.[4] The characteristic that leads to such activity is a relative hydrophobicity, and it has been shown by detailed studies that a hydrophobic patch in framework region 1 contributed to by two β-strands is important in binding to the polysaccharide antigen and that this binding is modified by sequences in the complementarity-determining region H 3.[5] (This specific structure does not pertain to cold agglutinins of other specificity, and the reason for their specificity and reactivity, while probably similar, is unknown.)

How Do Cold-Reacting Antibodies Arise?

Cold agglutinins arise in two settings, both perhaps due in part to the fact that B cells utilizing the VH4-34 gene segment normally represent a relatively large percentage of the population (6%–13% of all mature B cells). These cells probably account for the low titers (< 1/10) of cold agglutinin that can be found in the serum of normal individuals. Oligoclonal antibodies, usually in

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elevated titers, appear routinely in moderate titers that are not sufficient to cause significant hemolysis in infectious mononucleosis (with anti-i specificity) and mycoplasma (with anti-I specificity) infections and less commonly in cases of cytomegalovirus, chicken pox, etc. Monoclonal cold-reacting antibodies are an expression of paraneoplasia (benign monoclonal gammopathy) or immunocyte neoplasia including chronic lymphocytic leukemia (CLL) and a variety of lymphomas.[6] Trisomy (complete or partial) of chromosome 3 has been recorded in patients in whom cold agglutinin disease progressed to a lymphoproliferative disorder.[7] The titer of the antibody is, in all cases, a relative measure of the number of abnormal cells.

The Donath-Landsteiner antibody of paroxysmal cold hemoglobinuria (PCH) formerly arose as a cross-reacting antibody to an antigen on *Treponema pallidum* (or so it is thought) and was frequently seen in secondary and tertiary syphilis; this is now rare. It is most commonly encountered in children as a response to viral illness or immunization, much like immune thrombocytopenia in this group; in these cases, it does not persist, but the clinical syndrome it produces may be severe enough to cause death. In adults, it is usually an autoimmune antibody and may be associated with other evidences of autoimmunity.[8]

Diagnosis of Diseases Due to Cold-Reacting Antibodies

Cold agglutinins are readily detected as, being IgM, they agglutinate red cells directly. Three characteristics should be determined: specificity, titer, and thermal amplitude (the highest temperature at which the antibody reacts with red cells). These are readily done with standard techniques.

The detection of Donath-Landsteiner antibodies is much more problematical. Direct agglutination is often present but in relatively low titers (usually less than 1/64). The direct antiglobulin test (direct Coombs' test) with anti-IgG is negative as the antibody elutes from the cells during their preparation. The indirect antiglobulin test is usually ineffective as well but occasionally is positive.[9] The test attributed to Donath and Landsteiner (the bithermic hemolysis of normal red cells) is positive only when the titer in the serum is relatively high. The sensitivity of the test can be increased greatly by the use of red cells from patients with PNH, which are more sensitive to the hemolytic action of complement. The best technique, not generally available, is the use of radiolabeled anti-IgG, which is affixed at 4°C but removed by raising the temperature to 37°C.[10] By using this technique, we were able to identify two patients not detected by the PNH variant of the Donath-Landsteiner test. Many patients with unexplained immune hemolytic anemia, often with a negative direct antiglobulin test, probably have undiagnosed PCH.

Why Is the Degree of Hemolysis So Different Among Patients?

The degree of hemolysis varies among patients because the characteristics of the antibodies responsible for it are so highly variable.[11] In the case of cold agglutinins, the amount of antibody (roughly measured by the titer) and the affinity of the antibody (roughly measured by its thermal amplitude) are the most important determinants of hemolysis. These should be measured both in saline and with albumin added. Some antibodies are less hemolytic because they fix complement inefficiently; these antibodies are usually cryoprecipitable and the effect may have to do with structural changes that result in that characteristic.[12] Some antibodies are not inhibited by the residual C3 on the red cell surface, as most are, and this results in increased hemolysis. Although most antibodies are pentameric IgM, some patients produce considerable quantities of hexameric IgM, which is more efficient in fixing complement.[13]

The same general principles of titer and thermal amplitude apply to Donath-Landsteiner antibodies. Several explanations have been offered to explain why these IgG antibodies result in intravascular hemolysis when the supposedly more efficient IgM antibodies generally do not. Certainly, the agglutination effected by IgM antibodies inhibits the fixation of complement components in vitro, but how much of a role this plays in vivo is unclear. D-L antibodies are able to fix C4 (but not C3) in the cold phase, but again the in vivo importance of this is questionable. The antigens of the D-L antibodies are said to lie closer to the membrane surface, thus facilitating the fixation of complement. In fact, the reason for the greater lysis of D-L antibodies compared to cold agglutinins is not clear.

What Is the Treatment of Diseases Due to Cold-Reacting Antibodies?

A primary treatment of any syndrome of cold-reacting antibodies is keeping the patient warm. This sometimes involves discomfort and is difficult for many patients to maintain. Where possible, avoidance of winter by migration is advisable.

In the case of IgM cold agglutinins, antibody production is not suppressed by prednisone and it should be used only when the simultaneous presence of IgG antibodies is found. Antibody production can sometimes be suppressed by chemotherapy, but the rate of proliferation of benign clones is so small that this is

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often ineffective; obviously, if malignancy is present, its treatment will treat the cold agglutinin problem. More recently, the use of anti-CD20 (rituximab) has found some success[14] as has the use of fludarabine.[15] Some patients appear to benefit by small to moderate doses of erythropoietin (personal observation). Plasmapheresis can be used to remove antibody temporarily but is difficult to maintain for chronic treatment; great caution is required to be sure that the blood does not get cold during the procedure.[16]

The treatment of PCH is like that of any other autoimmune hemolytic anemia due to IgG, except that splenectomy has no place in the regimen as the spleen is not involved in hemolysis. Prednisone, chemotherapy, and anti-CD20 treatment have all been used.

The hemolysis in the hemolytic anemia of cold-reacting antibodies is entirely dependent upon the activation and fixation of complement. In the future, the use of anti-C5 reagents, as described in Section I, or other complement modifying measures should provide relief of the most taxing symptom.

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Eculizumab

Humanized Anti-C5 Monoclonal Antibody

Prop Inn; USAN

h5G1.1
5G1.1

Immunoglobulin, anti-(human complement C5 α-chain) (human-mouse monoclonal 5G1.1 heavy chain), disulfide with human-mouse monoclonal 5G1.1 light chain, dimer

CAS: 219685-50-4

EN: 239304

Abstract

Eculizumab (5G1.1) is a recombinant, fully humanized monoclonal antibody that inhibits the activation of terminal complement components. Eculizumab binds specifically to the terminal human complement protein C5, preventing the release of the inflammatory mediator C5a and the formation of C5b-9 by inhibiting the cleavage of human complement C5 to C5a and C5b. Preclinical studies have demonstrated that the antibody has high binding affinity for C5, blocks the formation of C5a and C5b-9, and protects mammalian cells from C5b-9-mediated damage. Favorable clinical results have been reported in patients with membranous nephritis, rheumatoid arthritis and paroxysmal nocturnal hemoglobinuria (PNH) and the antibody is in phase II clinical development for these disorders; phase I trials have also been conducted in dermatomyositis.

Introduction

There is increasing evidence that activated components of the complement system contribute significantly to the pathogenesis of a large number of acute and chronic inflammatory diseases including rheumatoid arthritis, atherosclerosis, acute myocardial infarction and vascular injury after cardiopulmonary bypass (1-5). Furthermore, complement plays a significant role in the pathogenesis of paroxysmal nocturnal hemoglobinuria (PNH), characterized by intravascular hemolysis and venous thrombosis, and associated with aplastic anemia. Patients with PNH have a deficiency in CD59 and CD55 proteins on the surface of erythrocytes, platelets and other blood cells, which normally protect cells from complement-mediated injury (6-8). Patients with PNH experience fatigue, dysphagia, pain and hemoglobinuria, and the disease is associated with about 50% mortality (9).

Strategies that inhibit the damaging effects of complement on disease pathogenesis have therefore become a major focus in pharmacological research. In particular, inhibition of the complement cascade at C5, the cleavage of which results in the generation of the potent inflammatory mediators C5a and C5b-9 (10), while allowing the upstream generation of C3b, essential for combatting infections and preventing the development of autoimmune diseases (11), would appear to represent a promising approach.

Eculizumab is a recombinant, fully humanized monoclonal antibody that inhibits the activation of terminal complement components. Eculizumab binds specifically to the terminal complement protein C5 and prevents the release of the inflammatory mediator C5a and the formation of C5b-9 by inhibiting the cleavage of human complement C5 to C5a and C5b (12). The antibody is being developed for chronic indications including rheumatoid arthritis, membranous nephritis, dermatomyositis, lupus nephritis and paroxysmal nocturnal hemoglobinuria (13).

Pharmacological Actions

Eculizumab, as well as Fab and scFv fragments thereof, was evaluated for binding to complement and inhibition of complement activation *in vitro*. Eculizumab had similar binding affinity for human C5 compared to the murine antibody (IC_{50} ~ 2 nM), as determined in an ELISA, and it inhibited human serum complement-induced lysis of porcine aortic endothelial cells (PAEC) at the same concentration as the murine antibody (12).

Pharmacokinetics

A double-blind, randomized, placebo-controlled study was conducted with eculizumab (8 mg/kg i.v. once or

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twice monthly) to evaluate the pharmacokinetics/pharmacodynamics in 117 patients with idiopathic membranous nephropathy (mean creatinine clearance = 111 ml/min; 24-h proteinuria = 9 g; protein/creatinine ratio = 5; serum cholesterol = 270 mg/dl; hemoglobin = 12.0 g/dl; serum albumin = 2.8 g/dl). Preliminary results were reported for 11 patients. Mean steady-state trough serum eculizumab levels were 18 μg/ml in the twice-monthly treatment group and 8 μg/ml in the monthly treatment group. Mean steady-state mid-dose interval levels (1 week postdose on day 119 of the study) were 47 μg/ml for patients treated twice monthly and 35 μg/ml for those treated once monthly. Urine levels of eculizumab at midtrial and at the end of the trial were below the level of quantification in 8 patients, and 3 patients excreted 65-400 μg/24 h of eculizumab. All eculizumab-treated patients demonstrated < 10% of the hemolytic activity of placebo-treated patients at 1 h postinfusion. Serum eculizumab levels > 38 μg/ml inhibited hemolysis in all patients. Treatment with eculizumab was found to be safe and well tolerated, with no hypersensitivity reactions noted (14).

Pharmacokinetics and pharmacodynamics were also examined in an open-label pilot study in 11 transfusion-dependent patients with PNH who received treatment with eculizumab (600 mg weekly for 4 weeks, then 900 mg every 2 weeks for 2 months). Peak and trough serum levels of eculizumab were measured 1 h after the initial dose and then at weeks 3, 4, 6, 10 and 12, and in 10 patients eculizumab levels were > 35 μg/ml from 1 h after the first dose to the end of the study period. In 1 patient, eculizumab levels were > 35 μg/ml for the initial 10 weeks but < 35 μg/ml at week 12. In 10 patients, serum hemolytic activity was 20% or less for the entire treatment period, representing complete blockade, with only 1 patient showing increased hemolytic activity at week 12, which corresponded with a decline in eculizumab levels to < 35 μg/ml (15).

Clinical Studies

The safety and biological activity of a single i.v. dose of eculizumab (0.1-8 mg/kg) were investigated in 40 patients with mild to moderate rheumatoid arthritis. Mean C-reactive protein (CRP) levels were significantly reduced at day 7 compared to baseline after the highest dose and returned to baseline by day 14. Patients treated at this dose showed a trend for reductions in the signs and symptoms of rheumatoid arthritis. Doses of 2 mg/kg and above resulted in > 80% inhibition of human complement C5 for at least 10 h, and a single dose of 8 mg/kg provided > 80% inhibition for 10 days, with a return to baseline levels after 17 days. Eculizumab was safe and well tolerated across patient groups (16).

Another study examined the safety, efficacy and pharmacokinetics of eculizumab following multiple doses in 209 patients with rheumatoid arthritis. Patients were randomized to receive placebo, eculizumab 8 mg/kg every 2 weeks, eculizumab 8 mg/kg every week x 5 weeks, then every 2 weeks, or eculizumab 8 mg/kg every week x 5 weeks, then monthly, and evaluated both after 3 months of treatment and 3 months after terminating treatment. After 3 months of treatment, the induction/monthly group met the primary endpoint of improvement in ACR20 score (44% *vs.* 18% on placebo), and both induction groups met the secondary endpoint of decrease in CRP. Baseline C5b-9 levels were correlated with increased CRP levels and therefore systemic inflammation. Baseline C5b-9 levels > 200 mg/ml were associated with clinical response to eculizumab. In cases of elevated C5b-9, an ACR20 response was obtained in 9% of patients in the placebo group, 33% of patients in the twice-monthly eculizumab treatment group, 57% of patients in the induction/monthly treatment group and 50% of patients in the induction/twice-monthly treatment group. The induction regimens completely suppressed complement-mediated serum hemolytic activity up to week 4. The half-life of eculizumab was 10-14 days (17-20).

A randomized, double-blind, placebo-controlled phase IIb clinical trial evaluated the efficacy and safety of eculizumab in 368 adult patients with mild to moderate active rheumatoid arthritis undergoing treatment with methotrexate and/or leflunomide. The patients were enrolled at 69 sites in the U.S. and Canada and were randomized to receive placebo, twice-monthly eculizumab (600 mg i.v. weekly for 5 weeks, then 600 mg once every 2 weeks) or monthly eculizumab (600 mg i.v. weekly for 5 weeks, then 600 mg monthly). The primary endpoint was improvement in ACR20 score after 6 months of treatment. Monthly administration of eculizumab following an induction period was associated with a statistically significant, moderate improvement in the ACR20 score compared to placebo at 6 months. The percentage of patients who achieved an ACR20 response was 34%, 24% and 22%, respectively, on monthly eculizumab, twice-monthly eculizumab and placebo. Monthly therapy was also associated with evidence of a long-term antiinflammatory effect, with a significant reduction in erythrocyte sedimentation rate at 6 months. The incidence of adverse events was slightly higher among patients given eculizumab every 2 weeks, although the incidence of injection-site reactions remained low (21).

A double-blind, placebo-controlled phase I pilot study was performed to determine the safety and tolerability of eculizumab (8 mg/kg weekly for 5 weeks, then once every 2 weeks) in 10 patients with dermatomyositis compared with placebo (n=3). After 2 months of treatment, results showed moderate improvements in skin score, physician global score and manual muscle test in the eculizumab group compared with controls. The placebo group showed deterioration of physician global score, but improvements in skin score and manual muscle test compared to baseline. Disease intensity measured by magnetic resonance imaging (MRI) and various parameters on skin biopsy were also improved in the treatment group compared with placebo. Eculizumab was safe and well

tolerated, with no significant differences in adverse events between treatment groups (22).

Alexion has sponsored phase II trials in patients with membranous nephritis, with results showing good tolerance. One trial did not reach the primary clinical efficacy endpoint of reduction in proteinuria after 4 months, while in the other trial an increase in remission rate and significant improvements in proteinuria, hyperlipidemia, hypertriglyceridemia and hypoalbuminemia were obtained after 12 months. The company also began a phase II study in lupus nephritis several years ago following the observation of positive effects on proteinuria in a study in patients with systemic lupus erythematosus (13, 23).

Further results regarding efficacy and safety were reported from the open-label pilot study in 11 transfusion-dependent patients with PNH treated with eculizumab (600 mg weekly for 4 weeks, then 900 mg every 2 weeks for 2 months). After 3 months of initial treatment, all patients were enrolled into an open-label extension study and went on to receive eculizumab 900 mg every 2 weeks for over a year. In the 12 months prior to entrance into the study, patients had received over 4 red blood cell transfusions, had detectable glycosylphosphatidylinositol (GPI)-deficient hematopoietic clone, and had a negative throat culture for *Neisseria meningitidis* and *Neisseria gonorrhoeae*. After the first 3 months of treatment, serum hemolytic activity was completely inhibited in the 10 patients who showed serum trough levels of eculizumab above 35 μg/ml; quality-of-life markers were also significantly improved, transfusion rates decreased and serum levels of lactate dehydrogenase (LDH), an indicator of hemolysis, were significantly reduced. Increased levels of PNH type III erythrocytes were thought to be due to prolongation of PNH red cell survival and confirmed that treatment with eculizumab inhibits complement-mediated hemolysis. The proportion of PNH neutrophils, monocytes and platelets did not change. The reduction in transfusions was greatest for patients (n=7) without thrombocytopenia. Biochemical markers of hemolysis showed dramatic improvement 1 week after beginning treatment with eculizumab. The most common adverse events were headache and upper respiratory tract infections, followed by influenza-like symptoms, rigors, dizziness, nausea, nasal congestion and joint aches. No episodes of thrombosis were reported. No adverse events were considered to be drug-related and although 2 patients had serious adverse events, all patients completed the 12-week study (15, 24-26).

Reports of the cumulative results after 6 and 12 months of eculizumab treatment showed that patients with purely hemolytic disease (macroscopic hemoglobinuria with a normal platelet count; n=6) had the best response to treatment, with type III erythrocytes increased from 39% to 67%, median transfusions reduced from 2.0 units/patient/month to 0 units/patient/month, and suppression of hemolysis. Four patients who did not become transfusion-independent were treated with additional subcutaneous erythropoietin with some improvement. Two patients reported breakthroughs in complement activity, demonstrated by increased lactate dehydrogenase levels, hemoglobinuria and symptoms. These symptoms resolved immediately after the next dose of eculizumab and patients were managed by reducing the dosing interval. The most commonly reported adverse events after 12 months of eculizumab treatment were flu-like symptoms, sore throat, headache and upper respiratory infection. Four serious adverse events were reported, but there were no study discontinuations as a result of adverse events and all patients elected to continue eculizumab treatment. It was concluded that eculizumab was well tolerated, decreased hemolysis and transfusion requirements, and improved symptoms of PNH for over 12 months (27, 28).

Conclusions

Eculizumab is a promising new monoclonal antibody that binds to human complement C5 and inhibits its cleavage by C5 convertase. Eculizumab has been granted orphan drug status for the treatment of PNH in both the U.S. and Europe. A pilot program has been completed in patients with PNH and plans are being finalized with the FDA for the next stage of development. Pilot programs have also been completed in patients with dermatomyositis and eculizumab is in phase II trials for rheumatoid arthritis and membranous nephritis.

Source

Alexion Pharmaceuticals, Inc. (US).

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CLINICS

Cumulative results from an ongoing extension study showed that Alexion Pharmaceuticals Inc.'s eculizumab reduced hemolysis and the need for transfusions in patients with paroxysmal nocturnal hemoglobinuria (PNH). Eleven patients were treated in an initial, 12-week pilot study, and all elected to participate in two consecutive one-year extension studies. Maintenance doses of 900 mg eculizumab every 12-14 days were sufficient to completely and consistently block complement activity in all patients, as evidenced by the lack of hemolysis. The significant reductions in red blood cell destruction seen in the pilot study were maintained throughout the extension phases. The mean and median transfusion rates decreased from 2.1 and 1.8 units/patient/month, respectively, during the 12 months prior to eculizumab therapy to 0.4 and 0.3 units/patient/month, respectively, through the 23- to 26-month treatment period. These reductions were significant. Among 11 patients with aplastic PNH, seven have been transfusion independent for at least the last six months of the study period following treatment with eculizumab alone or in combination with erythropoeitin. **Details:** Leonard Bell, CEO, Alexion Pharmaceuticals Inc., 352 Knotter Dr., Cheshire, CT 06410. Ph: (203) 272-2596.

Novadel Pharma Inc. began a pilot feasibility trial of a lingual spray version of zolpidem, a drug for treating insomnia. The study will test the pharmacokinetics of a range of doses of the drug. If it is successful, Novadel intends to file an investigational new drug application to commence an abbreviated clinical development program designed to support a 505(b)(2) submission with the FDA. The product utilizes the company's patented lingual spray drug delivery technology, which is intended to provide for more rapid onset of therapeutic activity. **Details:** Barry Cohen, VP bus. & new prod. dvlpt., Novadel Pharma Inc., 25 Minneakoning Rd., Flemington, NJ 08822. Ph: (908) 782-3431, ext. 2160.

Baxter International Inc. has suspended enrollment in its Phase II/III clinical trial of its Preflucel influenza vaccine, currently underway in Europe. Preliminary data from the study showed a higher than expected rate of mild fever and associated symptoms in subjects who received the vaccine. The incidence of fever and associated symptoms was also higher than that seen with other vaccines available on the market. Baxter has put further clinical studies, which were planned to begin in 2005, on hold until analysis of data from this study is complete. **Details:** Deborah Spak, Baxter International Inc., One Baxter Pkwy., Deerfield, IL 60015-4625 . Ph: (847) 948-2349.

Intercell AG and Scigen Ltd. will collaborate on the development of a new therapeutic vaccine for hepatitis B virus (HBV). The proposed vaccine will combine Scigen's already licensed PreS1-, PreS2- hepatitis B virus antigen (Sci-B-Vac) with Intercell's novel synthetic Immunizer IC 31 as an adjuvant to induce the type of immune response needed to overcome the infection. Under terms of the agreement, Intercell will formulate the vaccine for clinical development and both parties will collaborate in the development and commercialization of the product. The two companies will share development costs, resulting in joint ownership of any future product. Further financial details were not disclosed. **Details:** Katharina Wieser, Intercell AG, Campus Vienna Biocenter 2, 1030 Vienna, Austria. Ph: 44 0 20 7404 5959. Email: kwieser@intercell.com. Mark Compton, mng. dir. & CEO, Scigen Ltd., 152 Beach Rd., #26-07/08 Gateway East, Singapore 189721. Ph: 61 2 9234 1700.

The Vaccine Research Center at the National Institute of Allergy and Infectious Diseases (NIAID) is starting trials of an experimental vaccine against severe acute respiratory (SARS) virus. The vaccine will be tested on 10 healthy volunteers at the NIH Clinical Center (Bethesda, MD). The clinic will do periodic follow-up exams on each volunteer for 32 weeks. The primary goal of the study is to determine if the experimental vaccine is safe in people. A secondary goal is to assess how well the vaccine stimulates the immune system to produce antibodies and cellular immunity, in this case, focusing on the SARS spike protein. The spike protein protrudes from the virus' outer envelope and helps it bind to cells it infects. Instead of using weakened or inactivated virus, which is typical for vaccine development, the new vaccine is composed of a small circular piece of DNA that encodes the viral spike protein. Scientists modified the DNA to minimize the risk of it combining with the SARS virus or other viruses of the SARS type, called coronaviruses. Scientists expect that the DNA will direct human cells to produce proteins very similar to the SARS spike protein. The immune system should recognize these proteins as foreign and then mount a defense against them. If the vaccinated person ever encounters the actual SARS virus, his or her immune system will be primed to neutralize it. **Details:** Linda Joy, med. rel., National Institute of Allergy and Infectious Diseases, NIAID Office of Communications & Public Liaison, 6610 Rockledge Dr., MSC 6612, Bethesda, MD 20892-6612. Ph: (301) 402-1663.

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of FLVCR was also increased in cell lines that are used to study heme transport (e.g., intestinal (Caco-2, 13696 copies/ng, MFI 35), and hepatic cells (HepG2, 504 copies/ng, MFI 56)), or have increased heme utilization (e.g., cancer cell lines (n=5, mRNA range 502-1845 copies/ng; MFI 28-54)).

This is the first description of a mammalian heme transporter. FLVCR exports heme to protect CFU-E from heme toxicity. Our expression studies suggest that hFLVCR may also be important for heme transport in other tissues, e.g., the small intestine, where heme serves as the predominant source of human dietary iron.

Abstract# 153

Imatinib Mesylate Reduces Phlebotomy Requirements in Polycythemia Vera. Richard T. Silver. *Hematology/Oncology, New York-Presbyterian Hospital Weill Cornell Medical University, New York, NY, USA.*

Erythrocytosis is the clinical feature that separates polycythemia vera (PV) phenotypically from the other chronic myeloproliferative diseases and is the cause of many of its most serious complications. The mechanism for autonomous erythropoiesis in PV remains unknown, but a number of growth factors other than erythropoietin including stem cell factor (SCF) are also essential for erythroid progenitor cell proliferation and differentiation. SCF signals by inducing tyrosine phosphorylation of its receptor, c-kit, and cross-talk may also exist between c-kit and the erythropoietin receptor. We therefore sought to determine if the tyrosine kinase inhibitor, imatinib mesylate (IM), which is known to block SCF signaling through c-kit could inhibit the autonomous erythropoiesis of PV. Seven patients, four women and three men, with PV as established by the diagnostic criteria of the Polycythemia Vera Study Group, were studied. All had an increased red blood cell mass by direct measurement, and all causes of secondary erythrocytosis were excluded. Their ages ranged from 32 to 76 yrs, (median 55 yrs). Before IM therapy, disease duration ranged from two months to 2.5 yrs (median 1.5yrs, mean 1.4yrs) and the number of phlebotomies per patient ranged from 2 to 36 (mean 11.6, median 6). Four patients had been treated with phlebotomy alone, and three others additionally with either hydroxyurea (HU), interferon or anagrelide. Two of the seven patients had palpable splenomegaly (10cm below the LCM size). All patients were initially phlebotomized to a Hct less than 42%. The starting dose of IM was 400mg daily with a dose escalation of 100mg daily each week to a maximum of 800mg if the platelet count was above 600,000/μl at two weeks. All seven patients had suppression of erythropoiesis. One patient with palpable splenomegaly had a reduction in spleen size by 50% and one had complete regression after only one month of IM therapy. All patients had cessation of phlebotomy requirements within the first 2 months of IM therapy except for one who required one additional phlebotomy. The median duration on IM has been 87 days (mean 84). To date, three of the seven patients have had a reduction in platelet count below 600,000/μl. There was no significant change in the leukocyte count. Two patients developed dry scaly skin; in one, it was sufficiently severe to require discontinuation of IM. In summary, IM therapy suppressed erythropoiesis in seven PV patients, reduced spleen size in two of two and lowered platelet counts in three of seven. A longer duration of treatment may be necessary to influence thrombopoiesis and normalize spleen size. However, the present results suggest that IM by blocking c-kit (and/or PDGF-R) may be a useful agent in the management of PV and warrants a more extensive clinical trial.

Abstract# 154

Eculizumab, a C5 Complement-Blocking Antibody, Abolishes Hemolysis and Renders Hemolytic Patients with Paroxysmal Nocturnal Hemoglobinuria (PNH) Transfusion Independent. Peter Hillmen,[1] Claire Hall*,[1] Judith Marsh*,[2] Dupe Elebute*,[2] Christopher F. Mojcik*,[3] Scott A. Rollins*,[3] Stephen Richards*,[1] Russell P. Rother*,[3] *[1]Haematology, Leeds Teaching Hospitals NHS Trust, Leeds, United Kingdom; [2]Haematology, St. George's Hospital Medical School, London, United Kingdom; [3]Alexion Pharmaceuticals, Inc., Cheshire, CT, USA.*

Paroxysmal nocturnal hemoglobinuria (PNH) is characterised by intravascular hemolysis, venous thrombosis and is associated with aplastic anemia. The complement-mediated hemolysis is due to the deficiency of complement-regulatory proteins on the surface of PNH red cells, particularly the terminal complement inhibitor CD59. The high risk of venous thrombosis results from PNH platelet activation by complement. Therefore complement inhibition in PNH should control the hemolysis and reduce the risk of venous thrombosis. Eculizumab is a humanized monoclonal antibody that binds to C5 and inhibits terminal complement activation. Ten transfusion-dependent PNH patients have been entered into an ongoing open label Pilot study of eculizumab and all patients will have completed the study by November 2002. Patients receive 600 mg eculizumab by intravenous infusion over 30 minutes weekly for 4 doses and then 900 mg every other week. To date, 4 patients have completed therapy and all have been entered into an extension study. Response data for at least 4 weeks of therapy is available for 6 of the 10 patients. Eculizumab has been well tolerated with no serious adverse events. No episodes of thrombosis have occurred although follow-up is short and 4 of 6 patients are on warfarin. The biochemical parameters of hemolysis dramatically improved or normalised for all patients within a week of starting therapy. Mean LDH decreased from 4494 +/- 3380 U/L prior to treatment to 636 +/- 166 U/L following treatment while mean AST decreased from 106 +/- 66 U/L to 24 +/- 9 U/L during the same interval. In addition, a progressive increase in the PNH type III red cells occurred during the treatment period. While hemoglobinuria was common in this patient group before treatment, no patient has had an episode of hemoglobinuria since commencing eculizumab therapy and patients report a marked improvement in symptoms related to hemolysis, such as dysphagia and lethargy. One patient began treatment during a severe acute hemolytic episode when she was passing nearly black urine and within 24 hours there was a complete resolution of her hemoglobinuria. Patients who had purely hemolytic disease (macroscopic hemoglobinuria with a normal platelet count; n=3) appeared to have the best response to eculizumab with cessation of hemolysis and an increase in mean hemoglobin from 9.4 +/- 1.6 g/dL to 10.3 +/- 0.6 g/dL at four weeks, obtained without transfusion. These hemolytic patients without cytopenias also showed a more pronounced increase in the proportion of PNH red cells (mean increase equal to 62%) presumably due to a prolongation of PNH red cell survival. None of these patients have required transfusions to date. Cytopenic patients with laboratory evidence of hemolysis but minimal hemoglobinuria (n=3) had a marked improvement in hemolytic parameters but less effect on transfusion requirements. There was no change in the proportion of PNH neutrophils, monocytes or platelets in any of the patients whilst on eculizumab. In summary, the preliminary results from this pilot study demonstrate that eculizumab was well tolerated, decreased hemolysis and improved symptoms in PNH patients. This study suggests that blocking complement at C5 is an effective therapeutic strategy for hemolytic PNH resulting in control of hemolysis and a reduction in transfusion dependency.

Abstract# 155

TCR Sequencing: The Molecular Signature of Autoimmunity in Aplastic Anemia. Antonio M. Risitano*,[1] Spencer Green*,[1] Susan Sharpe*,[1] Christine O'Keefe*,[2] Weihua Zeng*,[1] Jaroslaw P. Maciejewski*,[2] Neal S. Young.[1] *[1]Hematology Branch, NHLBI - NIH, Bethesda, MD, USA; [2]Experimental Hematology and Hematopoiesis Division, Taussig Cancer Center, Cleveland, OH, USA.*

Acquired aplastic anemia (AA) is a bone marrow failure syndrome characterized by immune-mediated destruction of hematopoietic progenitors. Because the specific, pathogenic antigens in AA are unknown, our research strategy has included analysis of the variable beta (Vβ)-chain to determine T-cell clonality and as a surrogate for an antigen-driven immune response. A major goal has been the molecular identification of disease-specific T-cell clones. In AA, the distribution of Vβ family usage is altered, with predominance or even expansion of particular Vβ subfamilies. Using flow cytometry, we have identified CD8+ Vβ-family expansions in 40 AA patients. As assessed by CDR3-length analysis, oligoclonally distributed Vβ families were selected, and their CDR3 cloned and sequenced in order to create a library of AA-specific clonotypes. As proof of principle, three frequently expanded Vβ families were initially selected (Vβ2, Vβ3 and Vβ17) for further study, including inter-patient comparisons. RT-PCR was performed using a common constant beta (Cβ) primer and a Vβ-family specific oligonucleotide. Corresponding Vβ regions from healthy donors were cloned and sequenced to determine the normal level of CDR3 diversity. As anticipated from the enormous repertoire of TCR-β chains, in controls almost all individual colonies obtained from a single Vβ PCR product had different sequences. In contrast, PCR products from AA patients showed in all cases a high level of redundancy, with one or two CDR3 sequences accounting for more than 50% of the entire Vβ-specific repertoire, confirming the oligoclonal nature of the expansion. Major clones showed unique sequences for each patient; in some cases small subclones with limited amino acid substitutions from the major clonotype were also present. Perfect correspondence between CDR3 length analysis and sequence was observed. All clonotypic sequences were absent from normal controls, consistent with a putative pathogenic role for patient-specific Vβ expanded cells. In two patients, analysis of serial samples obtained in different stages showed good correlation between the quantity of the identified clonotypes and activity of disease, with the frequency much lower after successful immunosuppressive treatment. A PCR using clonotype-specific CDR3 primers was used to screen and compare a large cohort of patients sharing HLA-class I antigen or the same Vβ expansion (3 or 2). Cloning and sequencing of selected samples led to the identification of some new clonotypes; sequences with high homology to the original patient-specific clonotypes were also found in other cases but at a lower frequency. In conclusion, probably pathogenic T-cell clones are present in most AA patients, strong evidence for an underlying immune pathophysiology. Moreover, their frequency within the entire T-cell pool appears to correlate with disease activity. The significance of highly homologous CDR3 sequences among patients of similar HLA-class I antigen background deserves further investigation but is suggestive of common inciting antigens. Our data support a view of most acquired AA as marrow cell destruction due to antigen-driven oligoclonal T cell immune response, involving limited numbers of Vβ clonotypes, which may differ in antigen affinity, killing efficiency, and susceptibility to immunosuppressive therapy, but which likely share the same hematopoietic target.

HEMOGLOBINOPATHIES II

Abstract# 156

Anti-Inflammatory Therapy Ameliorates Endothelial Activation and Improves Microvascular Blood Flow in Transgenic Sickle Mice. Dhananjay K. Kaul,[1] Xiao-du Liu*,[1] Chris Bryant*,[2] John D. Belcher*,[2] Gregory M. Vercellotti,[2] Robert P. Hebbel.[2] *[1]Medicine, Albert Einstein College of Medicine, Bronx, NY, USA; [2]Medicine, University of Minnesota, Minneapolis, MN, USA.*

In transgenic sickle (β^s) mice, but not normal mice, hypoxia followed by reoxygenation (H/R) causes a distinct inflammatory response characterized by endothelial oxidant generation, leukocyte recruitment and microvascular flow abnormalities (Kaul and Hebbel, J Clin Invest 106, 2000), indicating that hypoxia-induced sickling is etiologic in this response. This is consistent with our hypothesis that endothelial activation and leukocyte recruitment secondary to reperfusion injury would play an important role in the pathophysiology of sickle cell disease. The leukocyte recruitment is secondary to up-regulation of specific adhesion molecules on vascular endothelium. Previous studies have shown that sulfasalazine, a powerful inhibitor of nuclear factor (NF)-κB, a transcription factor, causes a significant decrease in endothelial adhesion molecules, i.e., vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin in both sickle cell anemia patients and β^s mice (Solovey et al. Blood 97, 2001). However, the consequences of NF-κB inhibition on inflammatory response (i.e., leukocyte recruitment) to hypoxia/reoxygenation (H/R) have not been ascertained under in vivo microcirculatory

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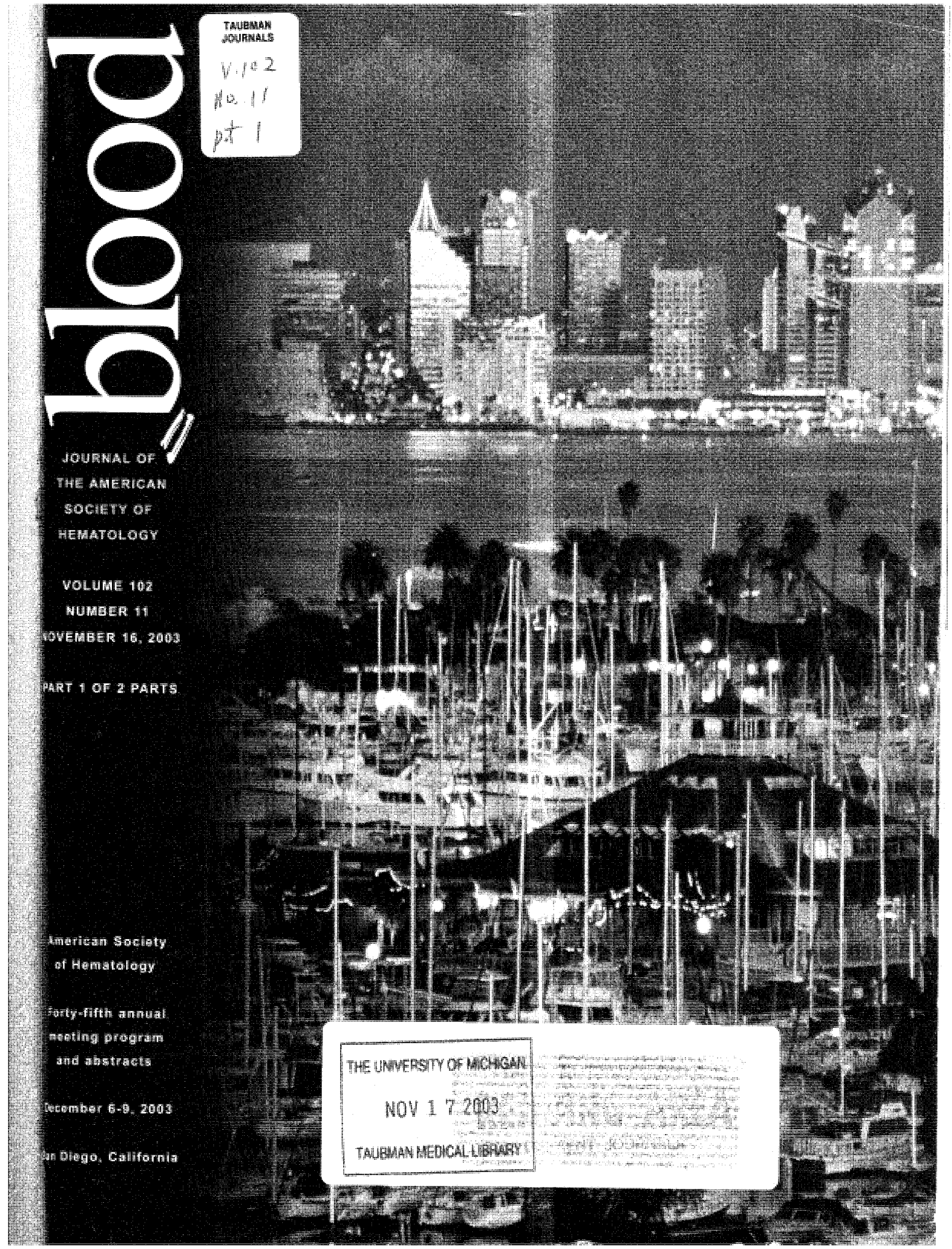
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vs. 8.7%, $P<0.001$) displayed significantly higher frequencies among AA patients than among a general Japanese population. The percentage of patients displaying DR15 was significantly higher in the 128 patients ≥30-years-old (77.3%) than in the 54 patients <30-years-old (37.0%, $P<0.001$). When all patients were divided into PNH$^+$ and PNH$^-$ groups, comprising 95 patients with a minor population of PNH-type cells and 62 patients without PNH-type cells, respectively, the increased frequency of DRB1*1502 was observed in both PNH$^+$ (23.9%) and PNH$^-$ patients (20.7%), while the increased frequency of DRB1*1501 was only observed in PNH$^+$ patients (21.2% vs. 6.7%). Both DRB1*1501 and the presence of PNH-type cells predicted response to monotherapy with cyclosporin A (CsA; odds ratios (ORs)=12.2 and 13.6, respectively), in addition to response to antithymocyte globulin (ATG) ±CsA therapy (OR=9.26 and 4.27, respectively). DRB1*1502 was not predictive of response to either therapy (OR=0.72 and 0.66, respectively). Multivariate analysis confirmed the predictive values of DRB1*1501 and the presence of PNH-type cells in both CsA therapy (P=0.047 and P=0.023, respectively) and ATG ±CsA therapy (P=0.048 and P=0.034, respectively). These data indicate that although both DRB1*1501 and DRB1*1502 contribute to the development of AA, their contributions may differ. Apparent age-dependent differences in HLA-DR15 frequency suggest that the pathophysiology of AA in adult patients may differ from that in pediatric patients. Furthermore, DRB1*1501 and the presence of a minor population of PNH-type cells represent independent factors favorably affecting response to IST in AA patients

Abstract# 1855 **Poster Board #-Session: 26-II**

Apoptosis and Proliferation of CD34 Positive Bone Marrow Cells of Polycythemia Vera Patients. Jie Bai*, Zonghong Shao, Hong Liu*, Jun Shi*, Guangsheng He*, Yanran Cao*, Meifeng Tu*, Yuhong Wu*, Liping Jing*, Tiejun Qin*, Hairong Jia*, Juan Sun*, Zhenzhu Cui*, Yanping Xue*, Huishu Chen*, Linsheng Qian*, Tanying Yang*, Chongli Yang*, *Institute of Hematology and Blood Disease Hospital, CAMS and PUMC, Tianjin, China.*

Objective To study the apoptosis and proliferation of CD34 positive(CD34$^+$) bone marrow cells(BMC) of patients with polycythemia vera(PV) **Method** The expression of Annexin V and proliferation related protein Ki67 in the CD34+ BMC of 20 PV patients and control cases[10 primary thrombocythemia(ET), 12 normal persons] were assessed by Bicolor flow cytometry(FCM), then the correlation between apoptosis and clinical situation was analysed in PV patients. **Result** The expression of Annexin V were (15.96%±1.45%)in PV patients' CD34$^+$ BMC(15.53%±1.76%)in ET patients'CD34$^+$ BMC which were lower than the expression in normal persons'.(23.61%±3.89%)($P<0.05$). The expression of Ki67 were(48.79%±11.68%) in PV patients' CD34$^+$ BMC and (49.60%±9.98%) in ET patients' CD34$^+$BMC which were significantly higher than that of normal control's(33.87%±6.82%). The ratio of apoptosiss/proliferation of PV patients was(0.33±0.104)and that of ET patients(0.32±0.02)which was significantly lower than that of normal control's. The apoptosis of CD34$^+$ BMC was negatively correlated with the level of hemoglobin(Hb)(r=-0.481,p=0.037), white blood cell(WBC)(r=-0.538,p=0.026) and the numbers of endogenous erythroid colony(EEC) of PV patients. The ratio of apoptosiss/proliferation of PV patients was negatively correlated with their Hb(r=-0.537, p=0.018)and WBC(r=-0.667, p=0.003). **Conclusion** There were lower apoptosis and higher proliferation in CD34$^+$ BMC of PV patients. Lower apoptosis was correlated with the disease severity of PV.

Abstract# 1856 **Poster Board #-Session: 27-II**

Flow Cytometric Analysis of Medullary Hematopoiesis in Tumor Necrosis Factor-α (Tg197) Mice: A Potential Model of Anemia of Chronic Disease. Renold J. Capocasale*,[1] J. O'Brien*,[1] D. Makropoulos*,[1] P. Rafferty*,[1] J. Quinn*,[2] R. Achuthanandam*,[3] M. Kam*,[3] L. Hrebien*,[3] P. Bugelski*.[1] (Intr. by Emmanuel C. Besa) *[1]Experimental Pathology, Centocor, Malvern, PA, USA; [2]School of Biomedical Engineering, Science, and Health Systems, Drexel University, Philadelphia, PA, USA; [3]Dept of Electrical and Computer Engineering, Drexel University, Philadelphia, PA, USA.*

Rheumatoid arthritis is a chronic inflammatory disease commonly associated with anemia of chronic disease (ACD). Heterozygous Tg197 transgenic mice expressing human tumor necrosis factor-α (TNF- α) develop a chronic progressive polyarthritis and weight loss and have been used as a model of rheumatoid arthritis (Shealy et al *Arth Res* 4:R7, 2002). As TNF-α is believed to play an important role in the pathogenesis of ACD, we used 4-color flow cytometry and routine hematology to evaluate the potential of Tg197 mice to serve as a model of ACD. Groups of 8 heterozygous Tg197 were euthanized at 5 or 10 weeks of age after 24 hr pulse labeling with BrdU. Age matched CBF1 mice served as controls. Hind limbs were collected and fixed in 10% formalin for paraffin sections. Blood and femoral bone marrow were collected for hematology and flow cytometry, respectively. Antibody to Sca-1 was used to evaluate hematopoietic stems cells, Ter-119/CD71 erythroid progenitors, Gr-1/CD11b granulocyte and monocyte progenitors and IL-7R-α, lymphoid progenitors. Annexin V binding, BrdU and 7-AAD were used to evaluate apoptosis and cell cycle in these populations. Histologic evaluation revealed a progressive arthritis and hematology a mild progressive, microcytic hypochromic anemia with reticulocytosis and monocytosis. Preliminary analysis of the flow cytometric data revealed increased numbers of dead and apoptotic erythroid precursors. The results of in-depth analysis will be presented. Taken together, our data suggest that Tg197 mice may be a useful model of ACD.

Abstract# 1857 **Poster Board #-Session: 28-II**

Relationship between Serum Erythropoietin and Hemoglobin Concentration in Community-Dwelling Older Women: The Women's Health and Aging Studies I and II. Paulo H. M. Chaves*, Qian-Li Xue*, Linda P. Fried*. (Intr. by Richard C. Woodman) *Johns Hopkins Center on Aging and Health, Baltimore, MD, USA.*

BACKGROUND. Anemia in older women is typically defined as Hb<12 g/dL. The current criteria used to define anemia were mainly derived from statistical distribution considerations. There is, however, limited physiologic data supporting or refuting these criteria. The objective of this study was to investigate the relationship between the continuous distributions of serum erythropoietin (EPO) – a surrogate marker for tissue oxygenation – and hemoglobin concentration (Hb) in older adults, focusing on the characterization of potential threshold effects. Specifically, we tested the assumption that within the Hb range defined as normal for older women by the World Health Organization (WHO); i.e., 12–16 g/dL, serum EPO is constant and lowest.

METHODS. This cross-sectional analysis used data from the Women's Health and Aging Studies (WHAS) I and II, two complementary population-based studies investigating the epidemiology of disability progression in 1002 women ≥ 65 yrs (WHAS-I, 1992-95), and disability onset in 436 high functioning women 70-80 yrs (WHAS-II, 1994-96). For this analysis, baseline WHAS I and WHAS II data from women 70-80 yrs were pooled (n=865). EPO was measured in sera samples stored at -70C by enzyme immunoassay. Within- and between-essays coefficients of variability for EPO were 13.3% and 11.6%, indicating acceptable reproducibility. Hb was measured by spectrophometry using the traditional cyanmethemoglobin method. Baseline EPO and Hb were available for 662 subjects. To minimize the impact of outliers, we restricted this analysis to subjects with Hb between 10–16 g/dL, and excluded 3 subjects with EPO values above the 99th percentile level (≥63 mU/mL). This study final sample size was 641. A generalized linear model with a log-link and gamma distribution was used to estimate the mean serum EPO per Hb concentrations, and EPO differences between different Hb concentrations with corresponding 95% confidence intervals (CI).

RESULTS. Median EPO was 7.1 mU/mL (25th – 75th percentiles: 3.1 – 13.6). Mean Hb was 13.2 ± 1.1 g/dL. Mean age of study group was 74.3 ± 2.9. The observed relationship between Hb and EPO was best described by a curvilinear, quadratic curve. EPO was highest at the lowest Hb, and monotonically decreased with increasing with Hb up to 14.2 g/dL (95% CI: 13.1, 15.3), the tangent to the curve. As compared to EPO at a Hb of 12 g/dL (11.3 mU/mL), mean EPO linked to a Hb of 10 g/dL (23.7 mU/mL) and 11 g/dL (15.4 mU/mL) were significantly higher; i.e., EPO was 2.1 (95%CI 1.4, 3.1) and 1.4 (95%CI 1.2, 1.6) times higher, respectively. Even within the normal Hb range, there was a substantial EPO gradient. For example, EPO at a Hb of 14.2 g/dL was .75 (95%CI .65, .88) times lower than at the low-normal Hb of 12 g/dL. Adjustment for age, race, and calculated creatinine clearance did not change these estimates.

CONCLUSION. We found a meaningful relationship between decreasing EPO and increasing Hb up to levels currently perceived as mid-normal Hb. These results support the hypothesis that mid-normal Hb concentrations might offer a clinical advantage over mild anemia (10-12g/dL) and possibly low-normal Hb (12-13 g/L). In addition, our findings are in accordance with recently published epidemiologic studies suggesting that a Hb of 12 g/dL might be a suboptimal criterion to define anemia in older women.

ERYTHROPOIESIS II

Abstract# 1858 **Poster Board #-Session: 29-II**

Eculizumab, a C5 Complement-Blocking Antibody, Controls Hemolysis in Paroxysmal Nocturnal Hemoglobinuria (PNH) with Responses Maintained over a Prolonged Period of Therapy. Peter Hillmen,[1] Claire Hall*,[1] Judith Marsh*,[2] Dupe Elebute*,[2] Stephen Richards,[1] Scott A. Rollins*,[3] Christopher F. Mojcik*,[3] Russell P. Rother*,[3] *[1]H.M.D.S., Leeds Teaching Hospitals NHS Trust, Leeds, United Kingdom; [2]Hacmatology,, St. George's Hospital Medical School, London, United Kingdom; [3]Alexion Pharmaceuticals, Inc., Cheshire, CT, USA.*

Paroxysmal nocturnal hemoglobinuria (PNH) is characterized by intravascular hemolysis and venous thrombosis. Deficiency of the terminal complement inhibitor CD59 from PNH red cells is the principle cause of the complement-mediated hemolysis. Eculizumab is a humanized monoclonal antibody that binds to C5 inhibiting terminal complement activation. We previously reported that eculizumab was very effective at acutely controlling intravascular hemolysis in a 3-month Pilot study of transfusion-dependent PNH patients. We now report that all 11 patients from the initial study entered an extension trial of 900mg eculizumab by i.v. infusion over 30 minutes every other week for one year. To date all patients remain on eculizumab, completing between 11 to 15 months of therapy. Results presented below represent cumulative data through 6 months. Eculizumab continues to be well tolerated with no dose limiting toxicity. The adverse events in the extension study are similar to those in the pilot study. The dramatic improvement in the biochemical parameters of hemolysis occurring in all patients within a week of starting therapy persists. Mean LDH decreased from 3111 +/- 598 U/L over the 12 months prior to treatment to 670 +/- 69 U/L over 6 months following treatment (p=0.003). Hemoglobinuria (daily urine color scale) decreased from 3.0 to 0.2 paroxysms/patient-month since starting eculizumab therapy with a significant improvement in quality of life measures related to hemolysis. In addition, PNH type III red cells progressively increased during the treatment period from a mean of 37% to 57% at 6 months (p=0.009). There was also a continued reduction in transfusion requirement from a median 1.8 units/patient/month in the 12 months prior to treatment to 0.4 units/patient/month over the last 6 months (p=0.001), and 5 of the 11 patients remain completely transfusion independent after a year of therapy. Patients with purely hemolytic disease (macroscopic hemoglobinuria with a normal platelet count; n=6) had the best response

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with cessation of hemolysis, an increase in type III red cells from 39% to 67% and a decrease in transfusions from a median of 2.0 units/patient-month to 0 units/patient-month. Four patients who did not become transfusion independent have been treated with subcutaneous erythropoietin in addition to eculizumab with some improvement in hemoglobin and transfusion requirement. Interestingly, in most patients LDH levels remain just above the upper limit, the bilirubin remains slightly to moderately elevated (under 50 umol/l), the haptoglobins remain undetectable and the reticulocytosis persists indicating a low level of well-compensated, presumably extravascular, hemolysis. Two patients experienced transient breakthroughs in complement activity associated with increased LDH, hemoglobinuria and mild symptoms; these resolved immediately upon next dose of eculizumab and further breakthroughs were prevented by a slight decrease in the dosing interval. These data support the role of terminal complement activation in hemolysis of PNH red cells and the mechanism of action of eculizumab in the control of hemolysis and symptoms in PNH. In summary, this study demonstrates that eculizumab is well tolerated, dramatically decreases hemolysis and transfusion requirement as well as improving the symptoms of PNH. The effectiveness of eculizumab has been maintained for a median of 12 months of therapy and is ongoing in all patients.

Abstract# 1859 **Poster Board #-Session: 30-II**

A Novel GATA-Specific Inhibitor (GSI) Rescues Anemia of Chronic Disease by Oral Administration. Shigehiko Imagawa,[1] Yoko Nakano*,[1] Naoshi Obara*,[1] Norio Suzuki*,[2] Toshiro Nagasawa*,[1] Masayuki Yamamoto.[2] *[1]Division of Hematology, Institution of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki, Japan; [2]TARA center, University of Tsukuba, Tsukuba, Ibaraki, Japan.*

The disorders associated with anemia of chronic disease (ACD) are characterized by the production of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), which increase the binding activity of GATA-2 and NF-κB and inhibit production of erythropoietin (Epo). Because Epo promoter activity is negatively regulated by GATA binding, GATA-2 may be responsible for impaired Epo synthesis in inflammatory disease *in vivo*. On the other hand, we found that N^G-monomethyl L-arginine (L-NMMA), which is increased in patients with chronic renal failure, inhibits NO and cGMP production, increases the binding activity of GATA and mRNA expression, and inhibits Epo promoter activity. Therefore, one common pathogenesis of ACD and anemia of renal disease appears to be *via* the stimulation of GATA binding activity. We have shown that intraperitoneal injection of K-7174, a GATA-specific inhibitor, improves Epo production which had been inhibited by IL-1β and TNF-α. In this study, a novel GSI, which has a 100-fold higher affinity than K-7174, was examined to further improve Epo production that had been inhibited by IL-1β and TNF-α. The addition of 100 nM GSI rescued the inhibition of Epo protein production and promoter activity induced by IL-1β and TNF-α, respectively. Electrophoretic mobility shift assays revealed that the addition of GSI decreased GATA binding activity, which was increased with the addition of IL-1β and TNF-α. Furthermore, intraperitoneal injection of mice with IL-1β or TNF-α decreased Epo levels, reticulocyte counts, hematocrits (Hts), hemoglobins (Hbs), the numbers of CFU-E from spleen and bone marrow, and the number of CD71+/Ter119+ cells from the spleen. However, oral administration of GSI reversed these effetcs. These results raise the possibility of using GSI as a novel drug for treating ACD by oral administration.

Effects of GSI on Hbs, Hts, reticulocyte counts, Epo levels, the number of CFU-E and CD71+/Ter119+ cells from ICR mice induced by IL-1β or TNF-α

	Control	+IL-1β	+TNF-α	+ IL-1β + 3 mg/kg GSI	+ TNF-α + 3 mg/kg GSI
Hb (g/dL)	14.8 ± 0.8	13.5 ± 0.5	13.4 ± 0.6	14.0 ± 0.8	13.8 ± 0.5
	(15)	(13) *	(11) *	(14) ***	(12) *****
Ht(%)	46.4 ± 2.8	41.6 ± 2.2	42.7 ± 2.0	43.4 ± 2.7	43.2 ± 2.1
	(16)	(13) *	(12) *	(14) ***	(12) *****
Reticulocyte (‰)	163 ± 29	122 ± 19	92 ± 32	304 ± 38	253 ± 55
	(10)	(8)*	(12) *	(8) ***	(12) *****
Epo (mU/mL)	24.8 ± 31.9	3.0 ± 4.6	3.3 ± 5.6	17.1 ± 24.1	49.4 ± 45.8
	(9)	(7) *	(8) *	(7) ***	(9) ******
CFU-E (spleen)	10.0 ± 8.9	4.7 ± 3.6	7.5 ± 4.7	30.4 ± 19.8	67.8 ± 7.9
	(8)	(6) *	(8) **	(8) ***	(8) *****
CD71+/Ter119+	19.6 ± 6.9	16.8 ± 6.9	ND	41.8 ± 11.6	54.3 ± 1.2
	(4)	(3)		(3) ****	(2)#

(experimental numbers) * Significance compared with control, P<0.005; **Significance compared with control, P<0.025; Significance compared with +IL-1β, P<0.005; ****Significance compared with +IL-1β, P<0.025; *****Significance compared with +TNF-α, P<0.005; #+TNF-α + 1 mg/kg GSI; ND:not done

Abstract# 1860 **Poster Board #-Session: 31-II**

Stress Erythropoiesis Is Not Efficiently Supported by a Minimal Epo Receptor Phosphotyrosine-Null Allele. Don M. Wojchowski,[PSU] Vinit G. Karur*,[PSU] Madhu P. Menon*,[PSU] Ke Li,[PSU] Jamie Crater*.[PSU] *[1]Department of Veterinary Sciences, The Pennsylvania State University, State College, PA, USA; [2]Pathobiology program, Department of Veterinary Sciences, The Pennsylvania State University, State College, PA, USA; [3]Molecular Medicine, Huck Institute of Life Sciences, The Pennsylvania State University, State College, PA, USA; [4]Department of Veterinary Sciences, The Pennsylvania State University, State College, PA, USA; [5]Department of Veterinary Sciences, The Pennsylvania State University, State College, PA, USA.*

Anemia intensifies signals for red cell production and activates stress erythropoiesis mechanisms. This can involve accelerated medullary erythropoiesis, enhanced cytokine action, stimulation of latent progenitor cell populations and extra-medullary erythropoiesis. To investigate ways in which subsets of Epo receptor linked factors might regulate this process, mice with a knocked-in truncated and phosphotyrosine-null EpoR allele (EpoR-HM) were challenged with phenylhydrazine. In these EpoR-HM mice, stress erythropoiesis was discovered to falter in several ways: 1) Hemoglobin levels, hematocrits and RBC numbers failed to efficiently rebound; 2) Interestingly, reticulocyte levels nonetheless approximated those in wt-EpoR controls; 3) In response to this hemolytic anemia, spleens of EpoR-HM mice essentially failed to enlarge; 4) Immunohistochemical analyses revealed decreased red pulp expansion in spleens of EpoR-HM mice; 5) Analyses of constituent erythroid progenitor cells revealed an apparent developmental deficiency in EpoR-HM mice at a late CD71-high, Ter119(+) stage; 6) In splenocyte preparations, proliferative responsiveness to Epo was several-fold lower for EpoR-HM mice, and markedly lower in the absence of serum; 7) Affymetrix profiling of purified Ter119(+) erythroid progenitor cells from spleen revealed elevated expression of several genes in EpoR-HM mice including an interesting set of GTP-regulated factors.These findings in general reveal that important roles are exerted by distal EpoR-PY-encoding domains during stress erythropoiesis (as illustrated, for example, by defective red cell production and splenic enlargement). In addition, insight into stress erythropoietic mechanisms also is advanced. This includes first a sustained deficit in mature red cells vs an efficient production of reticulocytes observed in EpoR-HM mice: this suggests that EpoR PY signaling normally may impact on reticulocyte half-life and/or maturation. Second, Epo-dependent stress erythropoiesis also may be significantly assisted in EpoR-HM mice by an accessory serum growth factor. Finally, profiling outcomes suggest that erythroid progenitor cell-intrinsic mechanisms also are activated in response to deleted EpoR-PY coupled events, and in part this may involve a selective engagement of GTP-protein linked pathways. Such novel stress erythopoietic mechanisms may have significant clinical relevance.

Abstract# 1861 **Poster Board #-Session: 32-II**

Complement-Mediated Hemolysis of Erythrocytes Is Blocked by RNA Aptamers to Complement Components C5 and C8. Jun-ichi Nishimura,[1] Shahid M. Nimjee*,[2] Haixiang Jiang*,[3] George Pitoc*,[2] Thad A. Howard*,[3] Christopher P. Rusconi*,[2] Mitsuhiro Omine*,[4] Michael M. Frank*,[3] Bruce A. Sullenger*,[2] Russell E. Ware.[3] *[1]Medical Oncology and Transplantation, Duke University Medical Center, Durham, NC, USA; [2]Experimental Surgery, Duke University Medical Center, Durham, NC, USA; [3]Pediatrics, Duke University Medical Center, Durham, NC, USA; [4]Medicine, Showa University Fujigaoka Hospital, Kanagawa, Japan.*

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hematopoietic stem cell disorder characterized by intravascular hemolysis, venous thrombosis, and bone marrow failure. The lack of GPI-anchored surface complement regulatory proteins, such as CD55 (DAF) but especially CD59, results in complement-mediated hemolysis and hemoglobinuria, which is a major clinical manifestation of PNH and an important cause of morbidity and mortality. Complement plays a critical role in the pathophysiology of hemolysis in PNH, and may also be important in the development of thrombosis. Accordingly, complement inhibition in PNH should control hemolysis and potentially reduce the risk of venous thrombosis. RNA aptamers that bind and functionally inhibit human complement protein C5 have been identified using a combinatorial RNA library and the systematic evolution of ligands by exponential enrichment (SELEX) in vitro selection method (Biesecker G. et al. : Immunopharmacology, 42:219, 1999). However, C5 is critical not only for complement activation, but also for proper regulation of inflammatory processes. Prolonged inhibition of C5 could, therefore, have undesired side effects that would limit its utility in patients with PNH. CD59 inhibits C9 from binding to the C5b8 complex, and is critically important in protecting erythrocytes from complement-mediated lysis. Individuals deficient in C9 are usually healthy, although inherited deficiencies in other complement proteins typically lead to various degrees of illness. Thus, complement proteins C8 or C9 may be the ideal targets to protect PNH erythrocytes from complement-mediated lysis without adverse side effects. We first synthesized one of the known C5-binding aptamers (UCUCAUGCGCCGAGUGUGAGUUUACCUUCGU) and confirmed inhibition of human serum hemolytic activity using an in vitro assay with sensitized sheep erythrocytes, C5-depleted serum, and purified C5. We have begun to identify RNA aptamers that inhibit complement components C8 or C9 by the SELEX procedure, to isolate high affinity, nuclease-resistant RNA ligands that bind specifically to C8 or C9. After only 4 rounds of the SELEX procedure, aptamers bound purified C8 with a Kd = 970 pM and also inhibited human serum hemolytic activity using an in vitro assay with sensitized sheep erythrocytes, C8-depleted serum, and purified C8. Complement-mediated lysis was inhibited at 50-100nM C8 aptamer concentrations, similar in potency to the published C5 aptamers. Additional rounds of the SELEX procedure should yield specific high-affinity C8-binding aptamers, and C9 aptamers are also being developed. Inhibition of complement activation is a new therapeutic goal for patients with PNH that could control hemolysis, reduce transfusion dependency, and potentially reduce the risk of thrombosis. The development of specific RNA aptamers by the SELEX procedure should provide new therapeutic options for patients with PNH in the near future.

Abstract# 1862 **Poster Board #-Session: 33-II**

Increased Apoptosis of Erythroid Cells during Anemia Recovery in Prion Protein Deficient (Prnp-/-) Mice. Jan H. Zivny*,[1,2] Karel Holada*,[2] Jan Simak,[1] Jaroslav G. Vostal.[1] *[1]Laboratory of Cellular Hematology, Center for Biologics Evaluation and Research, FDA, Bethesda, MD, USA; [2]1st School of Medicine, Charles University, Prague, Czech Republic.*

The cellular isoform of prion protein (PrPc) is involved in the pathogenesis of prion diseases in both humans and animals but its normal function is not known. It has been proposed that PrPc may protect cells against oxidative stress damage, promote survival and differentiation of neuronal cells and mediate copper transport. However, prion protein gene knock-out mice (Prnp-/-) did not develop major phenotypic changes. PrPc is expressed on many cell types including hematopoietic cells and red cells. We tested the role of PrPc in erythropoiesis using a mouse erythroleukemia cell line (MEL) induced to erythroid differentiation by N,N'-hexamethylene bisacetamide (5mM) and a mouse model of accelerated erythropoiesis induced by acute anemia. Erythroid differentiation of MEL cells led to the induction of prion protein gene (Prnp) mRNA expression and up-regulation of cell surface

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INHIBITION OF COMPLEMENT ACTIVITY BY HUMANIZED ANTI-C5 ANTIBODY AND SINGLE-CHAIN Fv.

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Abstract—Activation of the complement system contributes significantly to the pathogenesis of numerous acute and chronic diseases. Recently, a monoclonal antibody (5G1.1) that recognizes the human complement protein C5, has been shown to effectively block C5 cleavage, thereby preventing the generation of the pro-inflammatory complement components C5a and C5b-9. Humanized 5G1.1 antibody, Fab and scFv molecules have been produced by grafting the complementarity determining regions of 5G1.1 on to human framework regions. Competitive ELISA analysis indicated that no framework changes were required in the humanized variable regions for retention of high affinity binding to C5, even at framework positions predicted by computer modeling to influence CDR canonical structure. The humanized Fab and scFv molecules blocked complement-mediated lysis of chicken erythrocytes and porcine aortic endothelial cells in a dose-dependent fashion, with complete complement inhibition occurring at a three-fold molar excess, relative to the human C5 concentration. In contrast to a previously characterized anti-C5 scFv molecule, the humanized h5G1.1 scFv also effectively blocked C5a generation. Finally, an intact humanized h5G1.1 antibody blocked human complement lytic activity at concentrations identical to the original murine monoclonal antibody. These results demonstrate that humanized h5G1.1 and its recombinant derivatives retain both the affinity and blocking functions of the murine 5G1.1 antibody, and suggest that these molecules may serve as potent inhibitors of complement-mediated pathology in human inflammatory diseases.
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Key words: complement, C5, humanized antibody, Fab, scFv.

INTRODUCTION

Activated components of the complement system are believed to contribute to many of the pathophysiological mechanisms underlying numerous acute and chronic inflammatory processes (reviewed in Morgan (1994)). For example, cleavage of C3 generates both C3a, a relatively weak chemoattractant for neutrophils and C3b, a potent opsonin. The subsequent cleavage of C5 generates C5a, a potent chemo-attractant and activator of neutrophils, as well as C5b, which initiates deposition of the membrane attack complex C5b-9 on the cell surface. The recruitment of neutrophils contributes significantly to inflammatory tissue injury in numerous experimental systems (reviewed in Korthius and Granger (1994)), while C5b-9 deposition can result in cell activation due to calcium influx (Morgan, 1989), upregulation of cell surface adhesion molecules (Hattori *et al.*, 1989) or in cell lysis.

The complement cascade is regulated by endogenous inhibitors which act at numerous points including C1, C4, C3 convertase activity, C5 convertase activity and the formation of C5b-9 (Lachmann, 1991). Development of a soluble form of complement receptor type 1 (sCR1), an inhibitor of C3 convertase activity, has provided a means to examine the potential of complement inhibition for the amelioration of several disease processes including xenotransplant rejection (Pruitt *et al.*, 1994), lung and dermal immune complex-mediated injury (Mulligan *et al.*, 1992), experimental allergic encephalomyelitis (Piddlesden *et al.*, 1994), cardiopulmonary bypass (Moat *et al.*, 1992; Gillinov *et al.*, 1993) and reperfusion injury (Weisman *et al.*, 1990; Smith *et al.*, 1993; Pemberton *et al.*, 1993). However, inhibition of the complement cascade by sCR1 at the level of C3 convertase activity may be predicted to have significant clinical side effects. Generation of C3b is essential for the normal phagocytosis of bacterial and fungal pathogens as well as the clearance of circulating immune complexes (Liszewski and Atkinson, 1993). In fact, humans genetically deficient in C3 are subject to recurrent life-threatening infections and also suffer from a greatly increased incidence of autoimmune diseases such as systemic lupus erythrematosus and glomerulonephritis (Ross and Densen, 1984).

To avoid these complications, we have focused on the

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inhibition of the complement cascade at the level of C5, thereby blocking formation of the proinflammatory complement activation products C5a and C5b-9, while preserving the generation of C3b. Patients with a genetic deficiency of C5 suffer only from an increased incidence of Neisserial infections, which are in fact milder than infections in patients having normal levels of C5 (Ross and Densen, 1984). As there are no endogenous inhibitors specific for the C5 convertases, monoclonal antibodies were developed, which bind human C5 and prevent its cleavage by C5 convertase (Würzner *et al.*, 1991; Khroshus *et al.*, 1995). These antibodies inhibit complement-mediated acute cardiac tissue injury occurring in perfused heart models of xenotransplantation (Kroshus *et al.*, 1995) and block leukocyte and platelet activation in an *ex vivo* recirculation model of cardiopulmonary bypass (Rinder *et al.*, 1995). Additionally, in pigs, a monoclonal antibody against C5a reduces myocardial infarct size by 35% (Amsterdam *et al.*, 1995). Finally, a monoclonal antibody against mouse C5 (Frei *et al.*, 1987) blocks progression of established arthritis in a murine collagen-induced arthritis model (Wang *et al.*, 1995) and prevents development of glomerulonephritis in a murine model of systemic lupus erythromatosis (Wang *et al.*, 1995).

The desired pharmacological properties of complement inhibitors are dramatically different for acute versus chronic settings. In acute settings, a complement inhibitor should have a relatively short serum half-life, coupled with a rapid rate of tissue penetration, since complement activation occurs within the tissue (Schäfer *et al.*, 1986; Hugo *et al.*, 1990). Large molecules such as monoclonal antibodies have very slow rates of tissue penetration as compared to smaller molecules such as Fab fragments (Covell *et al.*, 1986) or single chain Fv (scFv) molecules (Yokota *et al.*, 1992). A complement inhibitor used in chronic settings should have a prolonged serum half-life and not provoke an immune response. In humans, murine monoclonal antibodies have a half-life of approximately 1 day (LoBuglio *et al.*, 1993) and uniformly provoke an immune response (Khazaeli *et al.*, 1994). These problems have been addressed by the development of humanized monoclonal antibodies which have serum half-lives of several days (LoBuglio *et al.*, 1989; Khazaeli *et al.*, 1991; Saleh *et al.*, 1992; Schüpbach *et al.*, 1993) as well as greatly diminished immunogenicity (LoBuglio *et al.*, 1993)

In this study, variants of the 5G1.1 monoclonal antibody have been engineered, which can be used in either acute or chronic settings. Firstly, the 5G1.1 variable regions were humanized using the CDR-grafting technique (Riechmann *et al.*, 1988). The humanized variable regions were then used to construct humanized antibody, Fab and scFv molecules which all maintained a high affinity for human C5 and blocked the generation of both C5a and C5b-9.

MATERIALS AND METHODS

Cloning of 5G1.1 *variable region genes*

For N-terminal amino acid sequencing, 75 μg 5G1.1 mAb was subjected to SDS–PAGE under reducing conditions and transferred to ProBlott membrane (Applied Biosystems, Foster City, CA, U.S.A.) as previously described (Evans *et al.*, 1995). Protein bands were localized by staining with Ponceau S, excised and subjected to amino acid sequence analysis using Edman chemistry, performed on a pulsed liquid protein sequencer (ABI model 477A) with the PTH amino acids analysed using an on-line microbore HPLC system (ABI model 120A).

To deblock the amino terminus of the 5G1.1 heavy chain, 10 mg 5G1.1 monoclonal antibody was exchanged into reducing buffer (5 M guanidine–HCl, 50 mM Tris–HCl, 10 mM dithiothreitol, pH 8.5), using a PD-10 column (Pharmacia, Piscataway, NJ, U.S.A.). After incubation for 1 hr at room temperature, 50 mM iodoacetamide was added and the incubation allowed to continue for 30 min. The carbamidomethylated light and heavy chains were then separated by size exclusion chromatography on a Superose 12 (Pharmacia) column equilibrated with 5 M guanidine–HCl, 50 mM Tris–HCl (pH 8.5). The purified light chain was exchanged into 50 mM sodium phosphate (pH 7.0), using a PD-10 column, digested using 0.5 mU pyroglutamate aminopeptidase (PanVera, Madison, WI, U.S.A.) per nmol of heavy chain protein and sequenced as previously described. For determination of internal amino acid sequence, the isolated light chain was exchanged into 2 M urea, 25 mM Tris–HCl, 1 mM EDTA (pH 8.0) and incubated at 37°C overnight with endoproteinase Lys-C (Promega, Madison, WI, U.S.A.) at a protease:protein ratio of 1:40. The digested material was run on a C18 reversed phase HPLC column (Beckman Instruments, Fullerton, CA, U.S.A.) and eluted using a linear acetonitrile gradient (0–50%) in 0.1% trifluoroacetic acid. Peaks were subjected to amino acid sequence analysis as previously described.

Cloning of the 5G1.1 heavy chain variable region (VH) and light chain variable region (VL) was initially performed using a set of commercially available primers (Mouse Ig-Primer Set, catalogue number 69831-1; Novagen, Madison, WI, U.S.A.) essentially as previously described (Evans *et al.*, 1995). Comparison to the obtained peptide sequences confirmed that the correct VH had been cloned, whereas neither of the two light chain amino acid sequences were present in the cloned VL. To isolate the 5G1.1 VL, the UWGCG program TFASTA was used to search the GenBank rodent subdirectory with the amino acid sequence obtained from the purified light chain (IQMTQSPASLSASVGETVT). An exact match to this sequence was located in the murine germline gene encoding the v-kappa k2 variable region (Seidman *et al.*, 1978). The DNA sequence of this germline gene was used to design an oligonucleotide for use as a variable region 5′-primer for isolation of the correct VL gene.

Humanization of 5G1.1 *variable regions*

Two humanized variants of the 5G1.1 VL and VH regions were constructed by CDR grafting (Reichmann *et al.*, 1988). For CDR grafting, the 5G1.1 heavy and light complementarity determining regions were intro-

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duced into the human heavy variable region H20C3H (Weng *et al.*, 1992) to yield h5G1.1VHC (Fig. 1A) or the human light variable region I.23 (Klein *et al.*, 1993) to yield h5G1.1VLC (Fig. 1B), respectively. Construction of each CDR-grafted variable region was performed by PCR amplification, using two synthetic overlapping oligonucleotides of approximately 200 nucleotides in length. The cloned PCR products were sequenced on both strands in their entirety to ensure the absence of PCR-introduced errors. Murine amino acids were introduced into the human framework regions at position 71 in the light variable region and positions 71 and 78 in the heavy variable region by PCR mutagenesis.

Expression and purification of Fab

Expression of chimeric and humanized 5G1.1 Fab molecules was achieved using the mammalian 293-EBNA expression system essentially as previously described (Evans *et al.*, 1995*b*). Briefly, a plasmid capable of producing a chimeric Fab was constructed by fusion of the murine VL to the human kappa (*Inv*3 allele) constant region and fusion of the murine VH to the human IgG1 (*G1m*(17) allele) Fd in the expression plasmid pAPEX-3P. Since the cloning strategy for the 5G1.1 VL did not obtain the leader sequence, the leader sequence of the human CD59 protein was cloned in frame to allow secretion of the chimeric light chain cDNA. Similarly, a plasmid capable of expressing the CDR grafted Fab, h5G1.1 Fab (CDR), was constructed by cloning the variable regions h5G1.1VHC and h5G1.1VLC into pAPEX-3P. Finally, a plasmid capable of expressing the CDR grafted Fab containing framework changes, h5G1.1 Fab (CDR + FW), was constructed by cloning the variable regions h5G1.1VHC+F and h5G1.1VLC+F into pAPEX-3P. 293-EBNA cells (Invitrogen, San Diego, CA, U.S.A.) were transfected with the pAPEX-3P expression plasmids and selected using 1 μg/ml puromycin. Secreted Fabs were purified using protein G Sepharose chromatography as described (Evans *et al.*, 1995*b*), dialysed into PBS, and stored at 4°C. Protein concentrations were determined by the Bradford assay (Bio-Rad, Melville, NY, U.S.A.).

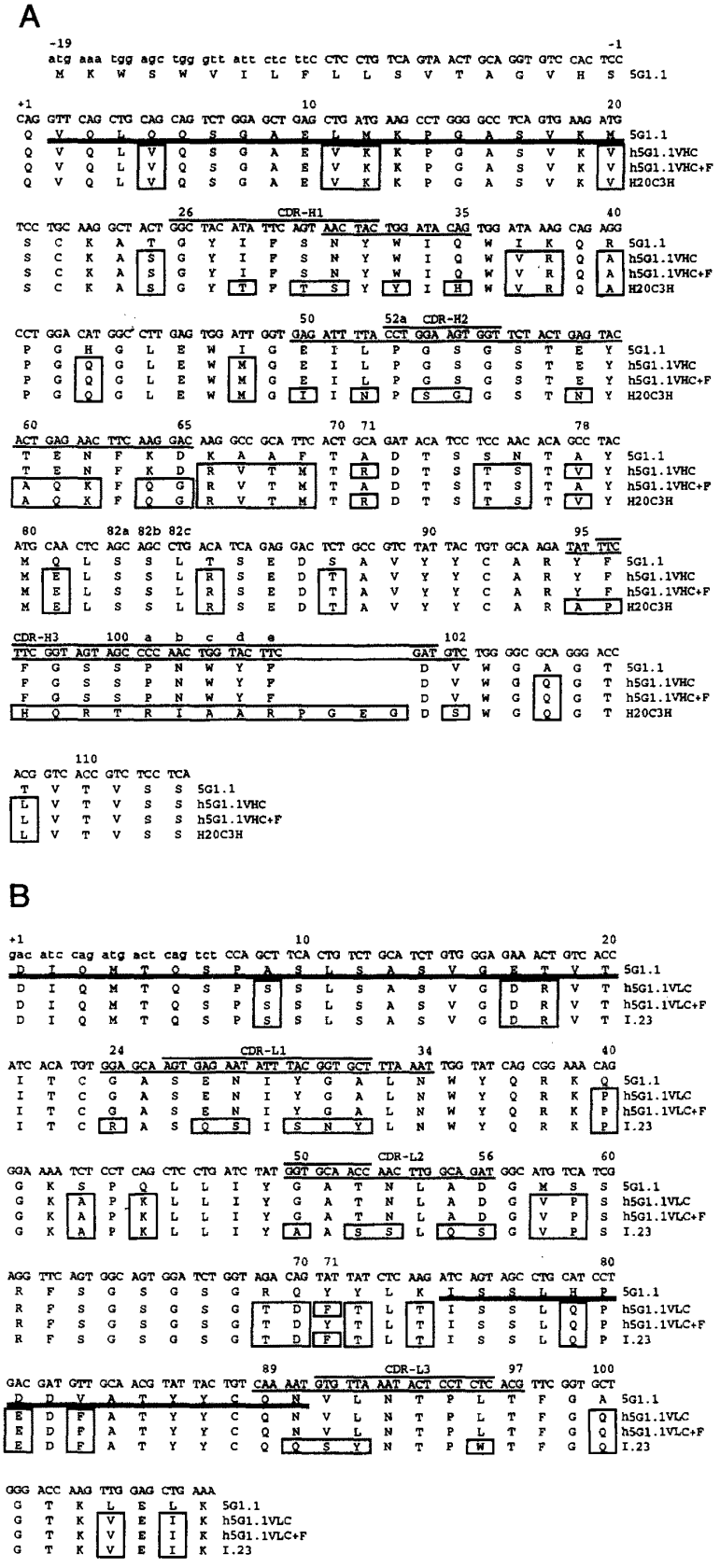
Construction of a humanized scFv

Two scFv proteins were constructed by the overlapping PCR technique. The h5G1.1 scFv (CDR) contained the h5G1.1VLC variable region, linked to the h5G1.1VHC variable region; while the h5G1.1 scFv (CDR + FW) contained the h5G1.1VLC+F variable region, linked to the h5G1.1VHC+F variable region. These scFv cDNAs were cloned into a plasmid in which expression is driven by the trc promoter. *Escerichia coli* strain ME1 was transformed with the resulting expression plasmids. Transformants were grown at 37°C in 2 l Applikon glass vessel fermentors containing Terrific Broth (1.2% (w/v) bactotryptone, 2.4% (w/v) bacto-yeast extract, 0.4% (v/v) glycerol, 90 mM potassium phosphate, pH 7.0), supplemented with 100 μg/ml ampicillin. The production of recombinant scFv was induced by the addition of 1 mM isopropylthio-β-D-galactoside when the OD_{550} of the culture reached 10. After an additional 3 hr incubation at 37°C, the cells were harvested by centrifugation and the cell pellets stored at −20°C. Cells were resuspended in 1 mM EDTA (pH 5.0), at 10 ml/g weight and lysed by a single pass through a microfluidizer M110-T (Microfluidics Corp., Newton, MA, U.S.A.). After centrifugation at 17 500**g** for 15 min, the resulting inclusion body pellet was resuspended in 20 mM Tris–HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl, 0.15% (w/v) deoxycholate at 10 ml/g inclusion body, using a Tekmar polytron. The inclusion bodies were again pelleted by centrifugation at 17 500***g*** for 15 min and resuspended in 20 mM Tris–HCl (pH 9.0) and 8 M urea at 10 ml/g. After stirring for 1 hr, the sample was centrifuged at 14 000***g*** for 30 min to pellet remaining insoluble material. The resulting supernatant was diluted 10-fold with 20 mM Tris–HCl (pH 9.0), 7 M urea and 50 μM cupric sulfate, and allowed to stir for at least 16 hr at 4 C to refold the scFv. Biocryl BPA-1000 (TosoHaas, Montgomeryville, PA, U.S.A.) was then added as a flocculation agent at 3 μl/ml (or at 10 μl/ml for the anti-mouse C5 scFv). After stirring for 5 min, the sample was centrifuged at 15 000***g*** for 10 min to pellet insoluble material. The supernatant was exchanged into 20 mM Tris (pH 9.0), 1 mM EDTA by diafiltration and concentrated by ultrafiltration using a stirred cell, fitted with a YM10 membrane (Amicon, Beverly, MA, U.S.A.). Properly refolded scFv was separated from aggregated material and contaminating proteins by anion exchange chromatography using Q Sepharose Fast Flow (Pharmacia). Bound scFv was eluted with 20 mM Tris–HCL (pH 9.0) and 1 mM EDTA containing a linear NaCl gradient (0–0.5 M). The fractions containing the scFv were combined, concentrated by ultrafiltration using a stirred cell fitted with a YM10 membrane and applied to a Sephacryl S200 HR 26/100 column (Pharmacia) equilibrated in 20 mM Tris–HCl, pH 9.0, 1 mM EDTA and 150 mM NaCl. Fractions containing the scFv were combined, exchanged into PBS by diafiltration, concentrated by ultrafiltration, filtered through a 0.22 mm Millex-GV filter (Millipore, Bedford, MA, U.S.A.) and stored at 4°C. Protein concentration was determined by the Bradford assay.

ELISA binding assays

For detection of recombinant Fab binding to human C5, F96 PolySorp microtiter plates (Nunc, Naperville, IL, U.S.A.) were coated overnight at 4°C with 0.1 mg/well human C5 (Quidel, San Diego, CA, U.S.A.) at a concentration of 2 mg/ml in 0.1 M Na_2CO_3, pH 9.6. The plates were then washed three times with 100 μl/well wash buffer (PBS containing with 0.5% (v/v) Tween 20) and blocked with 100 μl/well blocking buffer (PBS supplemented with 1% (w/v) bovine serum albumin, fraction V and 0.5% (v/v) Tween 20) at 37°C for 1 hr. The plates were again washed three times with wash buffer and incubated with 50 μl/well blocking buffer, containing 30 ng/ml murine 5G1.1 mAb, plus the indicated concentrations of inhibitor, at 37°C for 2 hr. The plates were

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again washed three times with wash buffer, and incubated with 50 μl/well blocking buffer, containing peroxidase-conjugated goat anti-mouse IgG Fc antibody, at a 2000-fold dilution (Sigma, St. Louis, MO, U.S.A.), at 37°C for 1 hr. After three final washes, the plate was developed with 50 μl/well substrate buffer (0.05 M phosphate–citrate buffer, pH 5.0, containing 0.3 mg/ml sodium perborate and 0.4 mg/ml *o*-phenylenediamine dihydrochloride). Reactions were stopped by the addition of 50 μl/well 1 M sulfuric acid and quantified using a Bio-Rad model 3550 plate reader set at 490 nm.

Hemolytic and C5a generation assays

The antibody, Fab, or scFv was pre-incubated with the indicated percent (v/v) human serum in 0.1 ml veronal buffered saline containing 1% gelatin ($GVBS^{++}$) for 30 min at room temperature. Chicken erythrocytes (Lampire Biological, Pipersville, PA, U.S.A.) were washed four times with $GVBS^{++}$ and resuspended at 5×10^7 cells/ml in $GVBS^{++}$, containing 1 μg/ml anti-chicken erythrocyte IgG (Inter-cell Technologies, Hopewell, NJ, U.S.A.). After incubation at 4°C for 15 min, the cells were washed twice with $GVBS^{++}$ and 3×10^6 cells were added to the pre-incubated human serum. After further incubation for 30 min at 37°C, the cells were pelleted by centrifugation at 10 000*g* for 2 min and the supernatant was assayed for released hemoglobin by measurement of the absorbance at 415 nm, and for C5a content by sandwich ELISA as described (Würzner *et al.*, 1991).

Porcine aortic endothelial cell (PAEC) lysis assays

PAEC were obtained from Cell Systems (Kirkland, WA, U.S.A.) at passage 1 and maintained in M199 medium, supplemented with 20% fetal calf serum, 20 mM HEPES, 10 μg/ml heparin (Sigma) and 5 μg/ml ECGF (Biomedical Technologies, Stoughton, MA, U.S.A.). PAECs at passage 3 were plated at 10 000 cells/well in a 96-well Falcon flat-bottomed microtiter plate. The following day, cells were washed twice with Hank's balanced salt solution, containing 1% (w/v) bovine serum albumin (HBSS/BSA) and incubated with 10 μM calcein AM (Molecular Probes, Eugene, OR, U.S.A.) in HBSS/BSA for 30 min at 37°C. Wells were washed twice with HBSS/BSA and incubated with 0.85 mg/ml rabbit anti-PAEC antibody (Kennedy *et al.*, 1994) for 30 min at 37°C. Wells were again washed twice with HBSS/BSA and incubated with HBSS containing 20% (v/v) human serum plus various concentrations of inhibitors for 30 min at 37°C. The supernatant was then transferred to a new 96-well Falcon flat-bottom microtiter plate and the remaining cells were solubilized with 1% SDS. The fluorescence of the supernatants and solubilized cells was determined using a Cytofluor 2350 (Millipore) with an excitation wavelength of 485 nm and an emmision wavelength of 530 nm. Percentage dye release was defined as the ratio of supernatant fluorescence to total (supernatant plus cell) fluorescence. Supernatant fluorescence in the absence of human serum was subtracted as background release.

RESULTS

Humanization of 5G1.1

The variable regions of the anti-C5 monoclonal antibody 5G1.1 were cloned by PCR amplification using 5′ primers, based on amino acid sequencing (for the light variable region) or degenerate 5′ primers (for the heavy variable region), coupled with 3′ primers specific for murine constant region sequences. Amino acid sequence analysis confirmed that the appropriate heavy and light variable regions had been cloned (Fig. 1). The 5G1.1 heavy variable region was most similar to V_H genes belonging to the V_H1/J558 family (Kofler *et al.*, 1992) and utilized the J_H1 gene. The most similar cloned murine V_H region was from the IgM secreting hybridoma 19.1.2 (Akolkar *et al.*, 1987). The 5G1.1 V_H differed from this clone at positions 20, 37 and 68 in framework regions, as well as at positions 28, 31 and 35 in CDR-H1, positions 58 and 65 in CDR-H2 and positions 95, 96 and 97 in CDR-H3. The 5G1.1 light variable region was most similar to the NYC V_κ gene (Jack *et al.*, 1992) and utilized the mouse $J_\kappa5$ gene (Hieter *et al.*, 1980), with amino acid differences present at framework position 72 in framework region 3 and at position 93 in CDR-L3.

For humanization, the 5G1.1 heavy variable regions CDRs were transposed into the human variable region

Fig. 1. Sequence of the 5G1.1 heavy (A) and light (B) variable regions. The DNA sequence and the translated amino acid sequence of the cloned 5G1.1 variable regions are shown. Amino acid position is numbered according to Kabat *et al.* (1992), with the complementarity determining regions according to the hypervariable sequence definition (Kabat *et al.*, 1992) or the structural variability definition (Chothia and Lesk, 1987) underlined and overlined, respectively. Lower case letters indicate nucleotide sequences derived from primers used for cloning. Amino acid sequences obtained from protein sequencing are indicated by double underlines. The protein sequences of the human variable regions H20C3H and I.23 are shown below the appropriate 5G1.1 variable regions. h5G1.1VHC and h5G1.1VLC denote humanized heavy and light variable regions constructed by grafting the CDRs from 5G1.1 on to the H20C3H and I.23 human framework regions. The variable regions h5G1.1 VHC+F and h5G1.1VLC+F contain murine amino acids at framework positions 71 and 78 in the heavy variable region and position 71 in the light variable region in addition to the murine CDR sequences. Amino acids in the human variable regions and the humanized 5G1.1 variable regions which differ from the murine 5G1.1 sequences are boxed.

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H20C3H (Weng *et al.*, 1992). This human V_H was derived from the human genomic V_H gene HG3 (Rechavi *et al.*, 1983), belonging to the V_H1 family and the human genomic J_H5 gene, and contains no changes in the framework regions from these genomic genes. The 5G1.1 light variable region CDRs were grafted into the human light variable region I.23 (Klein *et al.*, 1993). This human V_L region was derived from the human $V_\kappa I$ family gene O12 (Klein *et al.*, 1993) and the genomic $J_\kappa 1$ gene, with the introduction of an Arg residue in framework region 2 at position 38 in the I.23 cDNA as compared to the Gln residue encoded in the O12 genomic gene. Initial humanized 5G1.1 variable heavy and light regions were constructed by introducing the 5G1.1 CDRs into these human frameworks and are designated as h5G1.1 VHC and 5G1.1 VLC (Fig. 1).

Computer modeling of antibody variable regions has indicated that amino acid residues within the framework regions of a variable region can influence the structure of the CDRs. Comparison of the murine 5G1.1 variable region with the human acceptor variable regions H20C3H and I.23 suggested that the murine and human framework regions differed at three positions important for CDR structure. In the light chain, the presence of either Phe or Tyr at framework position 71 determines the canonical structure of CDR-L1 (Chothia and Lesk, 1987; Foote and Winter, 1992). Since I.23 contains a Phe residueat position 71, whereas 5G1.1 contains a Tyr residue, the Phe residue in h5G1.1 VLC was changed to a Tyr residue to create the variable region h5G1.1 VLC+F (Fig. 1). Similarly, the human H20C3H and murine 5G1.1 V_H regions differ at framework positions 71 and 78, which have been suggested to be important for maintaining the function of humanized antibodies (Carter *et al.*, 1992; Chothia *et al.*, 1989; Tramontano *et al.*, 1990; Foote and Winter, 1992). The murine amino acids were, therefore, introduced at these positions to create the variable region h5G1.1 VHC+F (Fig. 1).

Since choice of human antibody isotype can influence antibody avidity whereas isotype choice does not influence Fab affinity (Morelock *et al.*, 1994), initial analysis of the humanization procedure was performed using humanized Fab fragments. The murine 5G1.1 V_L and its humanized variants h5G1.1VLC and h5G1.1VLC+F were fused to the human kappa constant region (Inv3 allele), to create an intact light chain. The 5G1.1 V_H and its humanized variants h5G1.1VHC and h5G1.1VHC+F were fused to constant region 1 of human IgG1 (*G1m*(17) allele) to create an intact Fd. Three humanized Fabs were produced in 293-EBNA cells

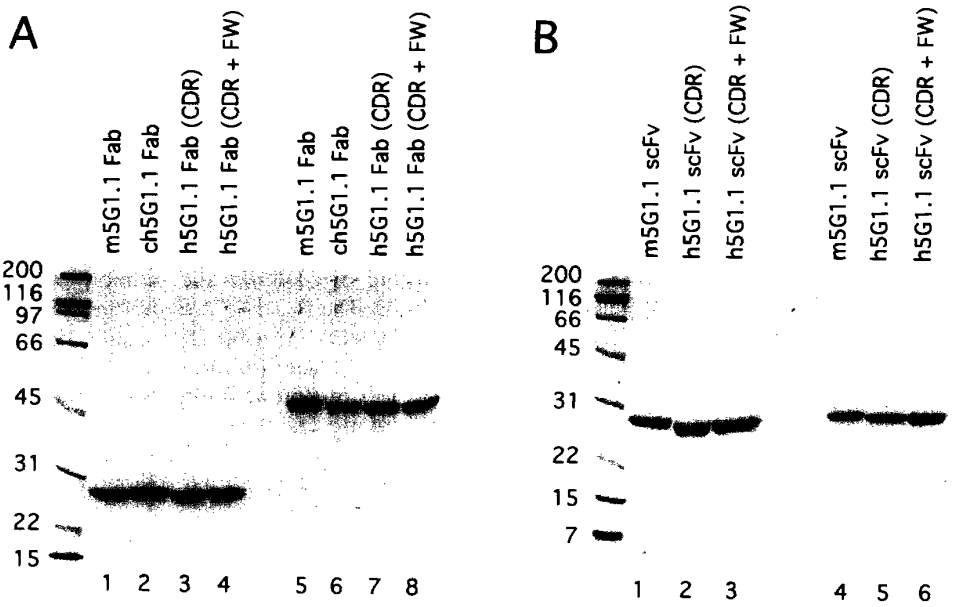


Fig. 2. SDS–PAGE analysis of purified recombinant proteins. (A) Fab fragments from the murine 5G1.1 mAb (m5G1.1 Fab) were produced by papain digestion of the m5G1.1 mAb followed by protein A chromatography to remove undigested antibody and Fc fragments. Recombinant chimeric Fab (ch5G1.1 Fab), CDR-grafted Fab (h5G1.1 Fab (CDR)), or CDR-grafted Fab containing murine framework amino acids (h5G1.1 Fab (CDR + FW)) were produced in 293 cells and purified from conditioned, serum-free medium using protein G-sepharose chromatography. Protein samples (10 mg) were subjected to SDS–PAGE under reducing (lanes 1–4) or non-reducing conditions (5–8) and stained with Coomassie R-250. (B) scFv molecules containing the murine variable regions (m5G1.1 scFv), the CDR-grafted variable regions (h5G1.1 scFv (CDR)), or the CDR-grafted variable regions containing murine framework amino acids (h5G1.1 scFv (CDR + FW)) were produced in *E. coli* and purified, as described in Materials and Methods. Ten micrograms of each purified scFv was subjected to SDS–PAGE under reducing (lanes 1–3) or non-reducing (lanes 4–6) conditions and stained with Coomassie R-250.

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by co-expression of light chain and Fd: (1) ch5G1.1 Fab, a recombinant chimeric Fab containing the murine 5G1.1 variable regions; (2) h5G1.1 Fab (CDR), a recombinant humanized Fab containing the h5G1.1VHC and h5G1.1VLC variable regions; and (3) h5G1.1 Fab (CDR + FW), a recombinant humanized Fab containing the h5G1.1VHC+F and h5G1.1VLC+F variable regions. SDS–PAGE analysis of the purified recombinant Fabs revealed a single band, which comigrated with Fab produced from 5G1.1 under non-reducing conditions and resolved into stoichiometric amounts of Fd and light chain bands, under reducing conditions (Fig. 2).

Binding of recombinant Fab and scFv to human C5

The affinities of Fab produced by papain digestion of the 5G1.1 mAb and the recombinant humanized Fabs were initially compared in a competitive binding ELISA, in which increasing concentrations of test Fab were used to displace murine 5G1.1 mAb binding to human C5 (Fig. 3). Biochemically produced Fab (m5G1.1 Fab) inhibited binding with an IC_{50} of approximately 2 nM. Similarly, the recombinant ch5G1.1 Fab, h5G1.1 Fab (CDR) and h5G1.1 (CDR+ FW) all inhibited binding by 50% at approximately 2 nM.

Although the humanized Fabs retained biological activity, neither the h5G1.1 Fab (CDR) nor the h5G1.1 Fab (CDR + FW) could be produced in significant quantities in *E. coli* (data not shown). Three scFv molecules were, therefore, constructed (Fig. 4); (1) m5G1.1 scFv, containing the murine variable light region and murine variable heavy region joined by a linker; (2) h5G1.1 scFv (CDR), containing the h5G1.1VHC and h5G1.1VLC variable regions; and (3) h5G1.1 scFv (CDR + FW), containing the h5G1.1VHC+F and h5G1.1VLC+F variable regions. These scFv molecules were produced as inclusion bodies in *E. coli*, refolded, and purified by anion exchange and sizing chromatography (Fig. 2). Gel filtration chromatography coupled with light scattering analysis demonstrated that at a concentration of 2.0 mg/ml (75 μM) the purified h5G1.1 scFv (CDR) was predominantly in the form of dimers. However, dilution of the h5G1.1 scFv resulted in a rapid shift of the equilibrium towards the monomer state (Thomas *et al.*, 1996). In the competition ELISA analysis, the m5G1.1 scFv inhibited binding of 5G1.1 mAb to human C5 with an IC_{50} value of 2 nM (Fig. 5), identical to the value obtained for the chimeric Fab (Fig. 3). Similarly, both the h5G1.1 scFv (CDR) and h5G1.1 scFv (CDR + FW) inhibited 5G1.1 mAb binding with IC_{50} values of 1.5 and 2 nM, respectively. In contrast, an scFv specific for mouse C5 (anti-mC5, Fig. 5) did not inhibit 5G1.1 binding, even at the highest concentrations assayed. Together these results indicated that the refolded scFv molecules bound to human C5 with an affinity equivalent to the Fab molecules produced in mammalian cells. Furthermore, no effect of the framework alterations could be discerned, based on the binding affinity of either the Fab or scFv molecules.

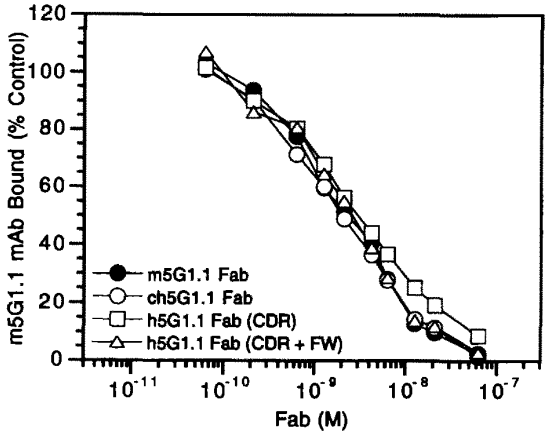


Fig. 3. Inhibition of 5G1.1 mAb binding to human C5 by recombinant Fab. 5G1.1 (0.2 nM) mAb plus the indicated concentrations of recombinant Fab were incubated together, for 2 hr at 37°C in microtiter plates coated with human C5. After washing, bound 5G1.1 mAb was detected using peroxidase-conjugated goat anti-mouse IgG (Fc specific) with quantitation performed by monitoring hydrolysis of *o*-phenylenediamine dihydrochloride at 490 nm. One hundred percent binding was defined in each experiment as the signal produced by binding of 5G1.1 mAb in the absence of competitor Fab. Data shown are mean values obtained from four to eight independent assays, with each datum point assayed in duplicate in each experiment.

Inhibition of C5b-9 formation by humanized Fab and scFv

The ability of the humanized Fab and scFv proteins to inhibit the formation of the C5b-9 membrane attack complex was evaluated using a hemolytic assay, in which the deposition of membrane attack complex on antibody-sensitized chicken erythrocytes is assessed by measuring hemoglobin release. Hemolysis was inhibited by greater than 90% when 0.25 μM of biochemically prepared murine 5G1.1 Fab, or each of the recombinant Fab molecules were added to the assay (Fig. 6A). This concentration corresponds to an approximate three-fold molar excess of Fab, as compared to the predicted human C5 concentration (0.08 μM) present in the assay. As with the Fab molecules, the scFv molecules also inhibited chicken erythrocyte hemolysis when present at a three-fold molar excess (Fig. 6B). The inhibition curves obtained with the scFv molecules were identical to those obtained with the Fab (Fig. 6B). The ability of the scFv to protect mammalian cells from complement-mediated damage was assessed by measuring their ability to block the lysis of antibody-coated porcine aortic endothelial cells (PAEC) by 20% human serum. The m5G1.1 scFv, h5G1.1 scFv (CDR) and h5G1.1 scFv (CDR + FW) all inhibited lysis of the PAEC when present at a three-fold molar excess, while the scFv specific for mouse C5 did not inhibit lysis, even at the highest concentration tested (Fig. 7A). The specificity of the 5G1.1 mAb for human C5 was maintained by the scFv, as neither the 5G1.1

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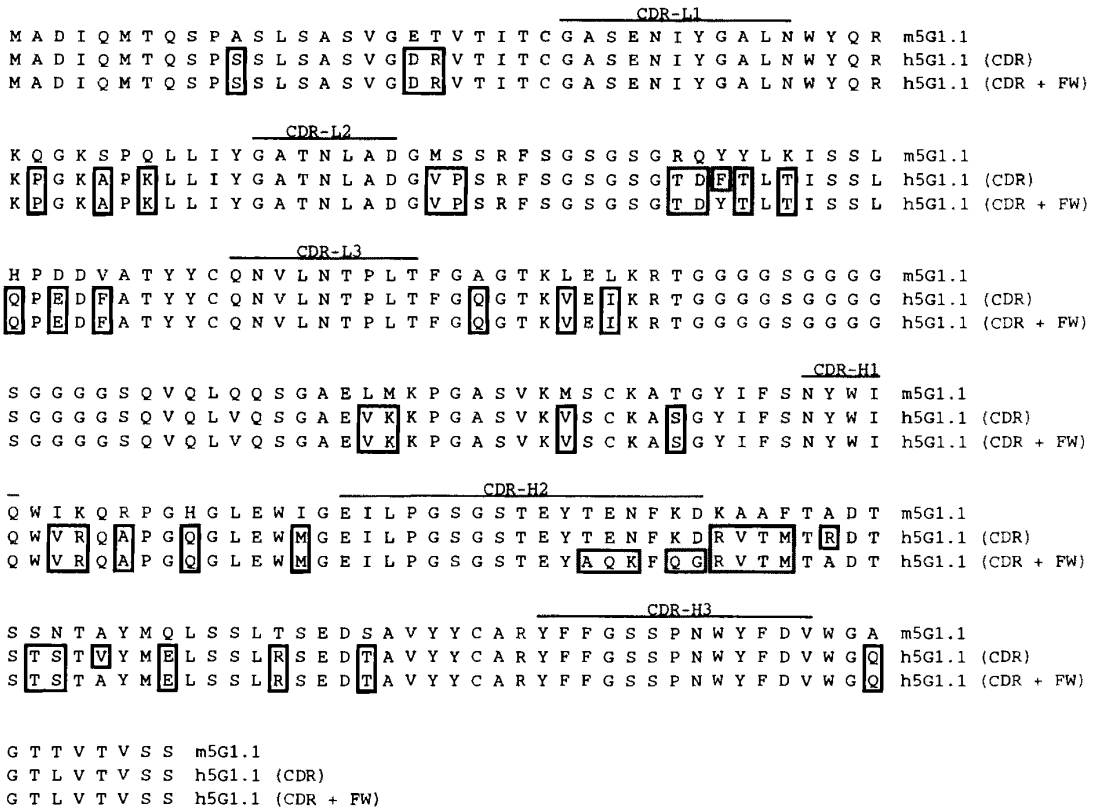


Fig. 4. Sequence of scFv molecules constructed using the 5G1.1 murine variable regions (m5G1.1) or humanized variable regions. The h5G1.1 (CDR) scFv contains the variable regions h5G1.1 VLC and h5G1.1 VHC, while the h5G1.1 scFv (CDR + FW) contains the variable regions h5G1.1 VLC+F and h5G1.1 VHC+F. The positions of the CDRs, as defined by Kabat *et al.* (1992), are indicated. Amino acid sequence analysis indicated removal of the initiator methionine, with the alanine residue at position two being the amino terminal residue of the bacterially produced scFv molecules. Amino acids in the humanized 5G1.1 variable regions which differ from the murine 5G1.1 sequences are boxed.

scFv, the h5G1.1 scFv (CDR) nor the h5G1.1 scFv (CDR + FW) could inhibit the lysis of PAEC by rat serum (Fig. 7B).

A critical parameter for use of an scFv as a C5 inhibitor is retention of the ability to block C5a generation. Previously, it has been found that a chimeric Fab, constructed from the monoclonal antibody N19-8 retained the ability to block both the formation of C5b-9 and generation of C5a, whereas an scFv constructed using the N19-8 variable regions retained the ability to block the formation of C5b-9, but only weakly inhibited the generation of C5a (Evans *et al.*, 1995). In contrast, analysis of supernatants obtained from the chicken erythrocyte hemolytic assays for C5a content (Fig. 8) indicated that equivalent concentrations of the humanized 5G1.1 Fab or scFv molecules blocked the generation of C5a. The concentration of inhibitor required for inhibition of C5a generation (Fig. 8) was comparable to the concentration of inhibitor required for inhibition of lysis (Fig. 6B).

Constuction of a humanized h5G1.1 antibody

Having demonstrated the effective humanization of the 5G1.1 variable regions, an intact humanized antibody (IgG4 isotype) was constructed and produced in 293-EBNA cells. The avidity of this humanized antibody (h5G1.1 HuG4) for human C5, was compared to the murine 5G1.1 mAb by determining the ability of each to compete binding of biotinylated 5G1.1 mAb to C5 (Fig. 9). The humanized h5G1.1 mAb had a two-fold lower avidity than the murine antibody. However, the humanized h5G1.1 HuG4 antibody was equipotent with the murine antibody at protecting PAEC from lysis by human serum, with a 0.5-fold molar ratio of antibody to C5 (1:1 ratio of antibody binding sites to C5) completely inhibiting lysis of the PAEC (Fig. 10).

DISCUSSION

This article describes the successful humanization of the anti-human C5 specific monoclonal antibody 5G1.1, utilizing the CDR-grafting technique. Although the original description of the CDR-grafting technique described the maintenance of antibody specificity by the transfer of the CDRs alone (Riechmann *et al.*, 1988), in most cases the transfer of a number of murine framework amino acids in addition to the CDRs has been found to be

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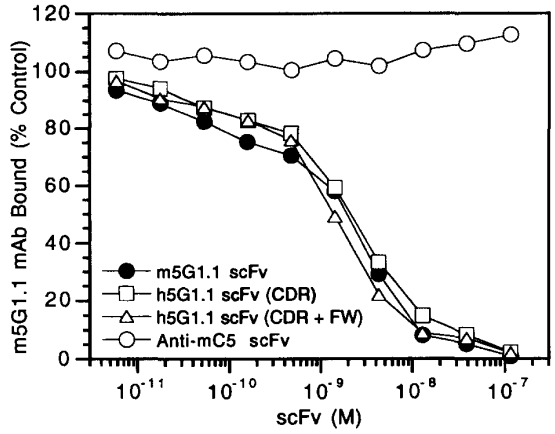


Fig. 5. Inhibition of 5G1.1 mAb binding to human C5 by recombinant scFv. 5G1.1 mAb (0.2 nM) plus the indicated concentrations of recombinant scFv were incubated together for 2 hr at 37°C, in microtiter plates coated directly with human C5. After washing, bound 5G1.1 mAb was detected using peroxidase-conjugated goat anti-mouse IgG (Fc specific) with quantitation performed by monitoring hydrolysis of *o*-phenylenediamine dihydrochloride at 490 nm. One hundred percent binding was defined in each experiment as the signal produced by binding of 5G1.1 mAb in the absence of competitor scFv. Data shown are mean values obtained from four to 10 independent assays, with each datum point assayed in duplicate in each experiment.

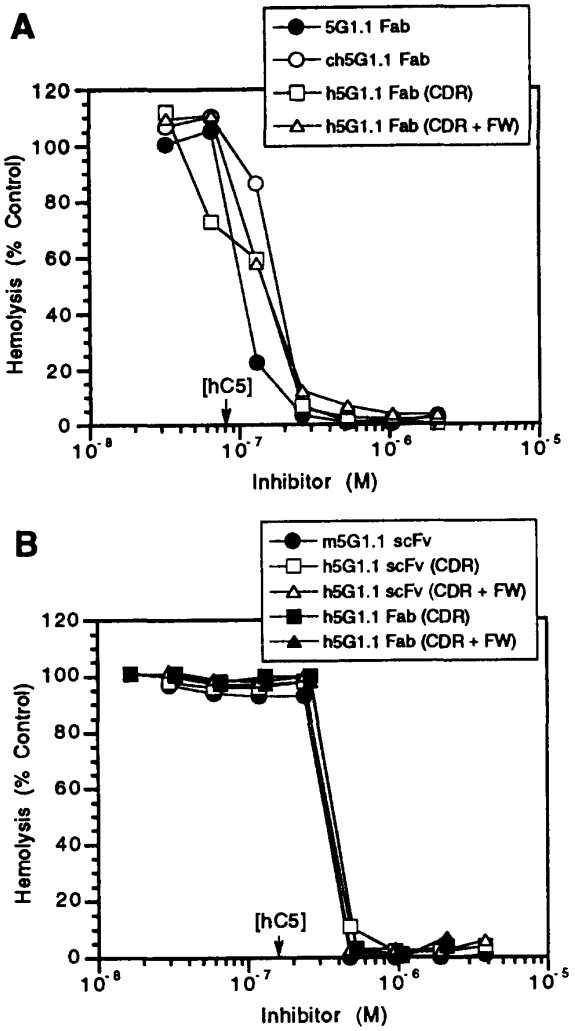


Fig. 6. Inhibition of chicken erythrocyte lysis by recombinant Fab and scFv. Twenty percent (A), or 40% (B), human serum in $GVBS^{++}$ was pre-incubated for 30 min at room temperature, with the indicated concentrations of competitors. Chicken erythrocytes, precoated with rabbit anti-chicken erythrocyte antibody, were added and the incubation was continued for 30 min at 37°C. The erythrocytes were then pelleted and the supernatants assayed for released hemoglobin by spectrophotometry (A_{415}). The A_{415} absorbance value obtained in the absence of inhibitor was defined as 100% hemolysis. Data shown are the average results of two to four independent determinations. The predicted hC5 concentrations in 20% and 40% serum are indicated by arrows.

necessary for retention of high affinity binding to the antigen (Winter and Milstein, 1991). These framework amino acids are believed to influence the structure of the CDRs. In particular, heavy variable region framework amino acid 71 determines the canonical structure of CDR-H2 (Tramontano *et al.*, 1990), while light variable region framework amino acid 71 determines the canonical structure of CDR-L1 (Chothia and Lesk, 1987; Foote and Winter, 1992). The human variable regions chosen for grafting of the 5G1.1 CDRs differed from the murine variable regions at heavy region framework position 71 and light region framework position 71. Therefore, humanized variable regions were constructed and designated h5G1.1 (CDR + FW), which maintained the murine amino acids at both of these positions.

The importance of these framework amino acids on the humanization process was initially assessed using recombinant Fab produced in 293 cells. Both the Fab containing the framework changes, h5G1.1 Fab (CDR + FW) and the Fab with unmodified human framework regions, h5G1.1 Fab (CDR), had the same binding affinity as murine Fab, when assayed by competition ELISA (Fig. 3). Similarly, no difference could be discerned between h5G1.1 Fab (CDR), h5G1.1 Fab (CDR + FW) and the murine Fab in their ability to inhibit human complement-mediated lysis of chicken erythrocytes (Fig. 6A). The h5G1.1 Fab (CDR) and h5G1.1 Fab (CDR + FW) were also equivalent in their ability to block the generation of C5a (Fig. 8). Similarly, bacterial-produced, refolded scFv, containing either human (h5G1.1 scFv (CDR)) or murine (h5G1.1 scFv (CDR + FW)) amino acids at these framework positions, had identical affinities as measured by ELISA (Fig. 5) and inhibited chicken erythrocyte lysis (Fig. 6), porcine aortic endothelial cell lysis (Fig. 7) and C5a generation (Fig. 8) with equivalent potencies. Therefore, although computer modeling suggests the importance of these framework amino acids in CDR canonical structure, amino acid changes at these positions had no effect on the functional activity of the humanized 5G1.1 molecules.

A clinically useful C5 inhibitor must block generation of both C5a and C5b-9, as both of these molecules have

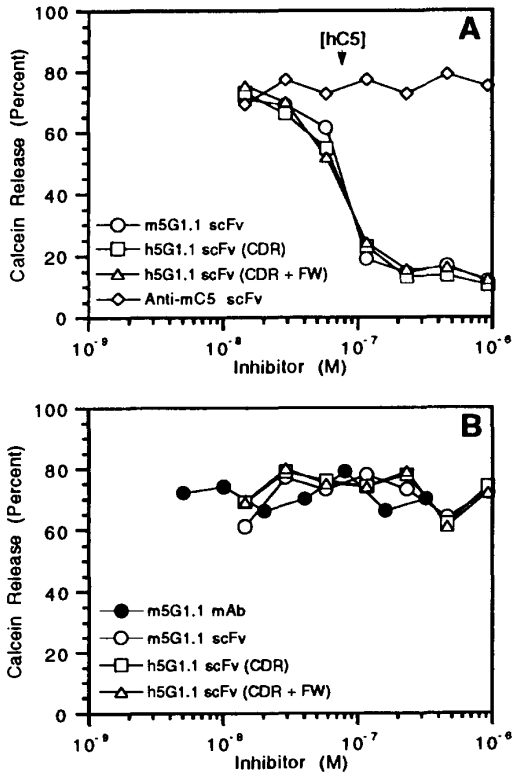


Fig. 7. Protection of porcine aortic endothelial cells (PAEC) from human serum complement by scFv. Calcein-labeled PAEC were incubated with HBSS containing 1% BSA and 0.85 mg/ml purified rabbit anti-PAEC polyclonal antibody for 30 min at 37°C. The cells were washed and incubated with HBSS containing 1% BSA plus 20% human serum (A), or 20% rat serum (B), and the indicated concentrations of inhibitor for 30 min at 37°C. Calcein dye release and retention were determined by measurement of the fluorescence of supernatants and SDS-solubilized cells, respectively, with dye release in the absence of human serum subtracted from all values. Results shown are the average of four to five independent determinations. The arrow denotes the predicted concentration of hC5 in 20% serum.

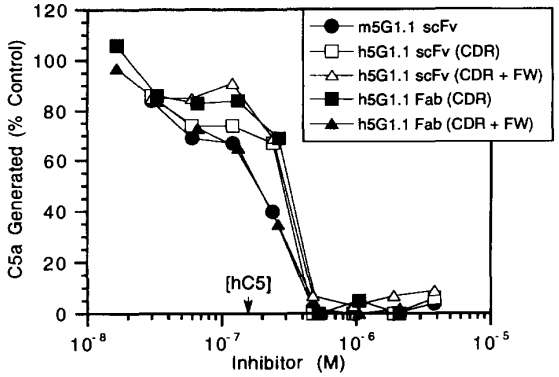


Fig. 8. Inhibition of C5a generation by recombinant Fab and scFv. Supernatants from chicken erythrocyte hemolytic assays performed using 40% human serum, as described in Fig. 5, were assayed for C5a content by ELISA. The C5a concentration obtained in the absence of inhibitor was defined as 100%. Data shown are from a representative experiment of several performed. The predicted hC5 concentration in 40% serum is indicated by the arrow.

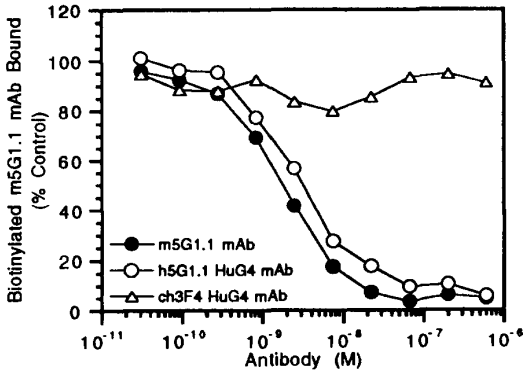


Fig. 9. Inhibition of 5G1.1 mAb binding to human C5 by recombinant humanized h5G1.1 HuG4 mAb. Biotinylated 5G1.1 mAb (0.2 nM), plus the indicated concentrations of unlabeled murine 5G1.1 mAb, recombinant h5G1.1 HuG4 mAb or a negative control recombinant HuG4 mAb (ch3F4 HuG4 mAb), were incubated together for 2 hr at 37°C in microtiter plates coated with human C5. After washing, bound biotinylated 5G1.1 mAb was detected using peroxidase-conjugated streptavidin, with quantitation performed by monitoring hydrolysis of *o*-phenylenediamine dihydrochloride at 490 nm. Absorbance values obtained in the presence of recombinant inhibitor were normalized in each individual experiment, to the value obtained in the absence of competitor. Results shown are mean values of four determinations.

potent pro-inflammatory effects. An scFv has been previously constructed from the variable regions of the monoclonal antibody N19-8 (Würzner *et al.*, 1991). The N19-8 antibody binds to human C5 with an avidity comparable to 5G1.1 (data not shown) and blocks generation of both C5b-9 and C5a. Although the N19-8 scFv has a high binding affinity for human C5 and inhibits the ability of human serum to lyse chicken erythrocytes, it only weakly inhibits C5a generation (Evans *et al.*, 1995). These findings suggest a model in which binding of the N19-8 mAb sterically blocks interaction of C5 with the C5 convertases. The N19-8 scFv may be too small to block cleavage by the C5 convertases, but remains bound to C5b and blocks formation of the C5b-9 complex. In contrast, the h5G1.1 scFv completely blocked generation of C5a when present at only a three-fold molar excess, relative to human C5 (Fig. 8). The 5G1.1 epitope may, therefore, be more directly involved in the interaction of C5 with the C5 convertases, than is the N19-8 epitope. Additionally, the h5G1.1 scFv maintains the ability to block the induction of both C5a- and C5b-dependent pro-inflammatory events in a closed loop model of cardiopulmonary bypass (S. A. Rollins, unpublished results).

Three important attributes of the humanized h5G1.1 scFv suggests its value as a therapeutic. Firstly, it main-

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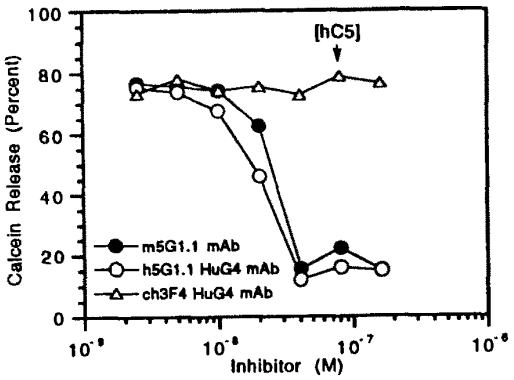


Fig. 10. Protection of porcine aortic endothelial cells from human serum complement by h5G1.1 HuG4 mAb. Calcein-labeled PAEC were incubated with HBSS containing 1% BSA and 0.85 mg/ml purified rabbit anti-PAEC polyclonal antibody for 30 min at 37°C. The cells were washed and incubated with HBSS containing 1% BSA plus 20% human serum and the indicated concentrations of inhibitor for 30 min at 37°C. Calcein dye release and retention were determined by measurement of the fluorescence of supernatants and SDS-solubilized cells, respectively, with dye release in the absence of human serum subtracted from all values. Results shown are the mean of three independent determinations. The arrow denotes the predicted concentration of hC5 in 20% serum.

tains the essential ability to block cleavage of C5 while preserving the immunoprotective properties associated with C3b generation. Secondly, it can be readily produced in large quantities in *E. coli*. Finally, the pharmacokinetics of scFv molecules are well matched to the time frame necessary for complement inhibition in acute settings where complement activation contributes significantly to the inflammatory process, such as cardiopulmonary bypass or myocardial infarction. Clearance of scFv molecules from the blood has previously been found to be extremely rapid with half lives of only a few minutes (Colcher *et al.*, 1990; Milenic *et al.*, 1991). At least two features of scFv molecules are likely to result in a rapid clearance rate, as compared to intact antibodies. Firstly, the prolonged plasma half-life of IgG is mediated through binding of the Fc region to specific "protection receptors", which have recently been demonstrated to be identical to the neonatal intestinal transport receptor (Ghetie *et al.*, 1996; Junghans and Anderson, 1996). Secondly, the small size of the scFv is well below the M_r 60 000 cut off of the glomerular basement membrane, resulting in the loss of the scFv into the urine. However, the binding of the scFv to C5 in the blood results in its incorporation into a high molecular weight scFv–C5 complex of nearly 220 000. Loss of bound scFv through glomerular filtration is, thus, dependent on the dissociation rate of the scFv from C5. Calculations based on the competitive ELISA data (Fig. 5), as well as direct measurements of h5G1.1 scFv (CDR) binding to human C5 by surface plasmon resonance (data not shown), indicated a very high affinity of the scFv for C5 ($K_d = 100$ pM) and a slow dissociation rate ($k_{off} = 1.0 \times 10^{-4}$/sec). This dissociation rate corresponds to an scFv–C5 complex half-life of approximately 2 hr. The 5G1.1 monoclonal antibody recognizes only human C5, precluding a direct assessment of h5G1.1 scFv pharmacokinetics in primates. In contrast, the N19-8 monoclonal antibody, which binds to human C5 with an avidity equivalent to 5G1.1, does bind to rhesus monkey C5. A single 100 mg bolus injection of N19-8 scFv into rhesus monkeys inhibits serum hemolytic activity for approximately 2 hr (Evans *et al.*, 1995b), further suggesting that the h5G1.1 scFv will have *in vivo* kinetic properties sufficient for use in acute clinical settings.

Complement activation has been suggested to be involved in the pathogenesis of several chronic human diseases including allograft rejection (reviewed by Baldwin *et al.* (1995)), systemic lupus erythematosus (reviewed by Mills (1994)), myasthenia gravis (reviewed by Drachman (1994)) and rheumatoid arthritis (reviewed by Morgan (1990)). Recently, it has been shown that prolonged treatment with an antibody to mouse C5 both blocks onset and disease progression in a collagen-induced arthritis model (Wang *et al.*, 1995), as well as inhibits development of glomerulonephritis in NZB/W mice (Wang *et al.*, 1996). As the half-life of the h5G1.1 scFv is likely to be too short to allow for chronic use in humans, a full length humanized antibody was also constructed. The human IgG4 isotype was chosen, as this isotype does not activate human complement (Tao *et al.*, 1993) and there is only one known allotype of IgG4 (Ghanem *et al.*, 1988), precluding the potential development of allo-antibodies in patients. The humanized h5G1.1 (CDR) HuG4 antibody bound to human C5 with a similar avidity as the murine antibody when assayed by ELISA (Fig. 9) and inhibited lysis of porcine aortic endothelial cells as effectively as the murine antibody, with a 1:1 molar ratio of antibody binding sites to human C5 being sufficient for inhibition (Fig. 10). Little information is available on the immunogenicity of CDR-grafted antibodies in humans. Repeated administration of chimeric antibodies containing intact murine variable regions, has induced an immune response directed against the murine variable regions in nearly all trials (reviewed in Khazaeli *et al.* (1994)). In this regard, it is significant that introduction of murine amino acids in the framework regions was not essential for maintenance of high affinity binding to C5. The h5G1.1 antibody is, therefore, likely to be minimally immunogenic in patients.

The recent successes of complement inhibitors in modulating numerous *in vivo* models of inflammation, suggest a great potential for complement inhibitors in the treatment of human inflammatory disease. The development of distinct C5-specific complement inhibitors with pharmacokinetic parameters, tailored for use in either acute or chronic settings, should facilitate the clinical assessment of the role of complement in such diseases.

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Datasheet for the decision of 20 September 2011

Case Number: T 1979/09 - 3.3.09

Application Number: 03000403.0

Publication Number: 1300084

IPC: A23L 1/30

Language of the proceedings: EN

Title of invention:
Composition for the treatment of chronic venous insufficiencies comprising an extract of red vine leaves

Patentee:
Boehringer Ingelheim International GmbH

Opponent:
Frutarom Schweiz AG

Headword:
-

Relevant legal provisions:
EPC Art. 83, 54, 56

Relevant legal provisions (EPC 1973):
-

Keyword:
"Inventive step (no - main and auxiliary requests)"

Decisions cited:
T 1416/07

Catchword:
-

Case Number: T 1979/09 - 3.3.09

D E C I S I O N
of the Technical Board of Appeal 3.3.09
of 20 September 2011

Appellant: (Opponent)
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Decision under appeal:
Interlocutory decision of the Opposition Division of the European Patent Office posted 14 July 2009 concerning maintenance of European patent No. 1300084 in amended form.

Composition of the Board:

Chairman: W. Sieber
Members: J. Jardón Álvarez
F. Blumer

Summary of Facts and Submissions

I. The mention of the grant of European patent No. 1 300 084, in respect of European patent application No. 03000403.0, in the name of Boehringer Ingelheim International GmbH, filed on 19 October 2000 as a divisional application of the earlier European patent application no. 00974420.2, was published on 19 April 2006 (Bulletin 2006/16). The granted patent contained 10 claims, whereby claim 1 read as follows:

"1. A composition in a form suitable for oral administration which consists of an active principle being capable of preventing or treating the discomfort associated with mild-to-moderate chronic venous insufficiency of the lower extremities and a pharmaceutically, cosmetically or dietetically acceptable carrier, the improvement wherein is that said active principle consists of an aqueous extract of red vine leaves containing 2 to 20% flavonoids, wherein said aqueous extract of red vine leaves is obtainable by a method comprising the steps of:

(a) collecting red vine leaves at a point of time when the content in flavonoids has reached an optimum;
(b) drying and crushing the leaves;
(c) cutting the leaves to pieces;
(d) extracting the leaves with water at temperatures from 60 to 80°C for 6 to 10 hours in an exhaustive percolation;
(e) optionally concentrating the obtained extract;

characterized in that said composition contains an amount of said aqueous extract of red vine leaves,

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which corresponds to a daily dosage of 300-800 mg of the extract."

Claims 2 to 10 were dependent claims.

II. A notice of opposition was filed by Frutarom Schweiz AG on 29 December 2006 requesting the revocation of the patent in its entirety on the grounds of Articles 100(a) (lack of novelty and lack of inventive step), (b) and (c) EPC.

During the opposition proceedings, *inter alia*, the following documents were cited:

D1: Ministère des Affaires Sociales et de la Solidarité, Bulletin Officiel, No 90/22 bis, "Médicaments à base de plantes", 1990, pages 24, 25, 36 and 37;

D4: Monographies de la 10e édition de la Pharmacopée Française et du Formulaire National (FN) en vigueur à la date du 1er janvier 1996, Extrait de vigne rouge (sec) (altogether 4 pages);

D5: Rote Liste 1998, Venentherapeutika, 83 048 and 83 084; "Antistax®";

D6: H. Beck, "Rotes Weinlaub von A bis Z", PTA heute, Nr. 8, August 1997, pages 792-796; and

D28: Frutarom Switzerland LTD, Versuchsprotokoll VENT, dated 11 July 2005 (6 pages).

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III. By its interlocutory decision announced orally on 17 June 2009 and issued in writing on 14 July 2009, the opposition division held that the grounds for opposition did not prejudice the maintenance of the patent in amended form on the basis of claims 1 to 9 according to auxiliary request 1.

Claim 1 of auxiliary request 1 was based on granted claim 1 (see point I above) wherein the expression "A composition" had been amended to read "A dietary supplement composition" based on granted claim 8.

In view of the experimental results filed by the opponent (cf. D28), the opposition division was convinced beyond reasonable doubt that the method of D4 provided an extract which was indistinguishable from the extract obtained by carrying out steps (a)-(d) of claim 1, even if D4 did not specify the extraction temperature and the flavonoid content of the extract. Notwithstanding the above, the opposition division acknowledged novelty because D4 neither disclosed a material being suitable for use as dietary supplement nor a carrier that was pharmaceutically, cosmetically or dietetically acceptable.

The opposition division considered D5 or D6 to represent the closest state of the art, because the Antistax® products disclosed in these documents were intended for the same purpose as the claimed product. The problem underlying the patent in suit was to provide an alternative product for oral administration being suitable for treating chronic venal insufficiency. The claimed solution using the extract according to claim 1 was seen as involving an inventive step because

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the skilled person would have regarded the presence of esculin in the Antistax® products of D5 and D6 as essential so that he would not have contemplated the exclusion of said component from the known products.

IV. On 14 September 2009 the opponent (appellant) lodged an appeal against the interlocutory decision of the opposition division. The appeal fee was paid on the same day.

The statement of grounds of appeal was filed on 12 November 2009 together with the following further documents:

D30: P.H. List and P.C. Schmidt, "Technologie pflanzlicher Arzneizubereitungen", Wissenschaftliche Verlagsgesellschaft mbH Stuttgart, 1984, altogether 29 pages;

D31: R.O.B. Wijesekera, Ed., "The Medicinal Plant Industry", CRC Press, Inc, 1991, Chapter 7, pages 85-88, 93-96 and 111;

D32: Les médicaments à base de plantes (Septembre 1997), Agence du Médicament, Paris, 1998; pages 3-9, 54-57, 60 and 61; and

D33: Copy of the minutes of the oral proceedings of 22 September 2009 in appeal case T 1416/07-3309.

The appellant requested that the decision under appeal be set aside and the patent be revoked in its entirety because the claimed subject-matter lacked novelty and inventive step, and was insufficiently disclosed. The

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appellant further requested that the point of law, which had been the subject in G 3/06, be again referred to the Enlarged Board of Appeal, because the referral proceedings in G 3/06 had been terminated following the withdrawal of the remaining appeals in the referring decision.

V. With its reply dated 28 May 2010 the patent proprietor (respondent) disputed all the arguments submitted by the appellant and requested that the appeal be dismissed.

It also filed the following fresh evidence:

D34: E. Schneider, "Gutachterliche Darstellung zur Aussagekraft von Leitsubstanzen als alleiniger Parameter zur Charakterisierung und zum Vergleich von Pflanzenextrakten unter Betrachtung der Phytoäquivalenz", dated 18 February 2008, 22 pages;

D35: M. Veit, "Stellungnahme zur Vergleichbarkeit pflanzlicher Extrakte *sui generis* und Gutachten zur Einordnung und Vergleichbarkeit von unterschiedlichen Weinlaubextrakten", dated 18 February 2008, 14 pages; and

D36: Pharmaton GmbH, Fachinformationen relating to Antistax® Kapseln, Januar 1996.

VI. On 1 April 2011 the board dispatched a summons to attend oral proceedings. In the attached communication the board drew the attention of the parties to the points to be discussed during the oral proceedings.

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VII. On 19 August 2011 both the appellant and the respondent filed further arguments in support of their requests.

The respondent also filed a set of seven claims for an auxiliary request marked "Auxiliary Request I- Appeal" and the following two further documents:

D37: PLANTA-SUBTIL Arzneimittel GmbH, "Zusammenfassung der pharmakologischen, toxikologischen und klinischen Untersuchungen zur Wirksamkeit von ANTISTAX Liquidum bei Venenerkrankungen (Chronisch Venöser Insuffizienz)"; a non-dated internal document; and

D38: C. Diehm, "Comparison of leg compression stocking and oral horse-chestnut seed extract therapy in patients with chronic venous insufficiency" THE LANCET, vol. 347, February 1996, pages 292-294.

VIII. Oral proceedings were held before the board on 20 September 2011 during which the appellant withdrew its request for a referral to the Enlarged Board of Appeal (see above point IV). The appellant requested that documents D37 and D38 not be admitted into the proceedings because they were *prima facie* not relevant and/or not published before the filing date of the patent in suit. During the oral proceedings the appellant's main and auxiliary requests were discussed.

The claims of the main request are the claims of auxiliary request 1 found allowable by the opposition division (see point III above).

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The claims of the auxiliary request are the claims filed on 19 August 2011 as "Auxiliary Request I - Appeal". Claim 1 of the auxiliary request reads as follows:

"1. A dietary supplement composition for use in the prevention or alleviation of the discomfort associated with mild-to-moderate chronic venous insufficiency of the lower extremities in a form suitable for oral administration which consists of an active principle being capable of preventing or treating the discomfort associated with mild-to-moderate chronic venous insufficiency of the lower extremities and a pharmaceutically, cosmetically or dietetically acceptable carrier, the improvement wherein is that said active principle consists of an aqueous extract of red vine leaves containing 2 to 20% flavonoids, wherein said aqueous extract of red vine leaves is obtainable by a method comprising the steps of

(a) collecting red vine leaves at a point of time when the content in flavonoids has reached an optimum;
(b) drying and crushing the leaves;
(c) cutting the leaves to pieces;
(d) extracting the leaves with water at temperatures from 60 to 80°C for 6 to 10 hours in an exhaustive percolation;
(e) optionally concentrating the obtained extract;

characterized in that said composition contains an amount of said aqueous extract of red vine leaves, which corresponds to a daily dosage of 300-800 mg of the extract, and the composition is administered in a

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daily dose of 300-800 mg of the extract in 1 to 3 capsules or tablets taken once daily."

IX. The arguments presented by the appellant in its written submissions and at the oral proceedings insofar as they are relevant for the present decision may be summarized as follows:

- The requirements of Article 83 EPC were not fulfilled, essentially because the specification did not describe a method for analyzing the complete group of flavonoids.

- The claimed subject-matter lacked novelty in view of D4. D4 disclosed the use of an auxiliary substance which could not be distinguished from a carrier.

- Concerning inventive step of both requests, the appellant pointed out that the situation in the present appeal case was the same situation as in the parent patent, which had been revoked for lack of inventive step (T 1416/07). The reasons given in that decision equally applied to the now claimed subject-matter.

X. The arguments of the respondent may be summarized as follows:

- The subject-matter of claim 1 was novel over the disclosure of D4 at least because there was no disclosure of a carrier as claimed. Additionally, the process of D4 did not result in the same extract as the one obtained when carrying out steps (a) to (e) of claim 1.

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– Regarding inventive step the respondent saw the disclosure of Antistax®, D5, as the closest prior art document. The subject-matter of claim 1 differed from this prior art in that the composition did not include esculin; the red vine leaves extract was specified to contain 2 to 20% flavonoids; and in that the method of preparation of the extract was specified. It pointed out that the experiments of the appellant, D28, did not show any prior art extract and that it was the task of the appellant to prove that the extraction conditions mentioned in D4 would inevitably result in an extract being identical to the claimed extract. The respondent stressed that there was no suggestion in the prior art that the red vine leaves extract alone would be sufficiently active. The fact that this extract would have the same efficiency as the known product Antistax® was an unexpected result. To affirm the contrary could only be done with knowledge of the invention (*ex post facto*).

– The auxiliary request involved an inventive step because there was no hint in the available documents as to the claimed dosage regime. On the contrary, the prior art's constant teaching (D5, D36) was towards an intake two or three times daily, actually teaching away from the invention. In the respondent's opinion it was quite unexpected that good results in treating chronic venous insufficiency could be obtained by using a different red vine leaves extract, eliminating the esculin from the Antistax® composition and administering it only once daily. Moreover, a single daily

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administration dosage regime was more likely to be complied with by the patients.

XI. The appellant (opponent) requested that the decision under appeal be set aside and that the European patent No. 1 300 084 be revoked.

The respondent (patent proprietor) requested that the appeal be dismissed (main request) or, subsidiarily, that the patent be maintained on the basis of "Auxiliary Request I - Appeal" as filed with letter dated 19 August 2011.

Reasons for the Decision

1. The appeal is admissible.

2. *Procedural matters*

2.1 The respondent filed documents D37 and D38 with letter dated 20 August 2010. The reason given for the late filing was that it had managed to get access to the unpublished study on Antistax® (D37) only during the preparation of its latest submission. D38 was necessary to properly understand the effects presented in D37.

2.2 The appellant requested not admitting these documents into the proceedings because they were *prima facie* not relevant and/or not published before the filing date of the patent in suit.

2.3 In the present appeal proceedings both parties filed new documents, namely D30 to D38. There is no evidence

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that the filing of these new documents by the respective parties amounts to a tactical abuse of the proceedings. The submissions were made at an early stage of the appeal proceedings or at the moment the documents were made available to the party. Moreover, the documents were filed in support of previous arguments and/or to establish the skilled person's general knowledge. Finally, the other party had enough time to take them into consideration. As regards the fact that D37 was not pre-published, the board notes that this document was merely used as a witness document to provide evidence for the effect allegedly achieved by the claimed subject-matter.

2.4 Thus the board saw no reasons not to admit the newly filed documents, and in particular D37 and D38 into the appeal proceedings (Article 114(2) EPC).

MAIN REQUEST

3. *Interpretation of Claim 1*

3.1 Claim 1 is directed to a dietary supplement composition with the following features:

(α) a dietary supplement composition
(β) in a form suitable for oral administration which consists of
(γ) an active principle being capable of preventing or treating the discomfort associated with chronic venous insufficiency
(γ1) the active principle consists of an aqueous extract of red vine leaves obtainable by a method comprising the steps of collecting red vine leaves,

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drying and crushing the leaves, cutting them to pieces and extracting with water under certain conditions, and

(δ) a pharmaceutically, cosmetically or dietetically acceptable carrier wherein

(ε) said composition contains an amount of aqueous extract of red vine leaves, which corresponds to a daily dosage of 300-800 mg of the extract.

3.2 Concerning feature (ε), it is noted that the expression "daily dosage" is a feature relating to the use of the composition, namely the amount to be taken per day, not to the composition itself. This feature is therefore not a limiting feature of the claimed composition. It is interpreted as indicating that the composition must be suitable for administering a daily dosage of 300-800 mg of the aqueous extract.

3.3 This interpretation of the claim is in conformity with the respondent's own interpretation (page 2 of its letter dated 19 August 2011).

4. *Sufficiency of disclosure*

4.1 The appellant has maintained during the appeal proceedings the objection that the requirements of Article 83 EPC are not satisfied because of the absence of a method for analyzing the complete group of flavonoids in the specification of the patent in suit.

4.2 In fact, the same objection has been raised in the appeal proceedings dealing with the parent application/patent, namely T 1416/07. As set out in point 3.5 of the reasons of this decision, "… it is

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well known which flavonoids are present in red vine leaves ... and that methods for analysing them, for instance high pressure liquid chromatography, are also well known and available to the skilled person without undue burden.... In these circumstances, the fact that it may be difficult and time consuming to identify and quantify all of the flavonoid species contained in the red vine leaves extract is not a matter to be criticised under the aspect of sufficiency." These reasons apply *mutatis mutandis* to the present case, in particular because the appellant has not provided any evidence whatsoever which could challenge the finding in T 1416/07.

4.3 Consequently the board is satisfied that the patent discloses the invention in a manner sufficiently clear and complete for it to be carried out by a person skilled in the art.

5. *Novelty (Article 54 EPC)*

5.1 The novelty of Claim 1 was contested by the appellant having regard to the disclosure of D4.

5.1.1 Document D4 discloses a method of preparation of a dry extract of red vine leaves (see under "Extrait de vigne rouge (sec), lines 1-10). According to D4, appropriate auxiliary substances can be incorporated, if necessary, before the drying step (lines 5-6).

5.1.2 However, the generic disclosure of "auxiliary substances" in D4 cannot take away the novelty of the specific carriers (pharmaceutically, cosmetically or dietetically acceptable carriers) now required in

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claim 1, in particular because D4 does not disclose any use of the extract for a pharmaceutical, cosmetic or dietetic application.

5.1.3 For this reason alone the subject-matter of claim 1 of the main request is novel.

6. *Inventive step (Article 56 EPC)*

6.1 The present invention relates to a dietary supplement composition having as active principle an extract of red vine leaves, for preventing or alleviating the discomfort associated with mild-to-moderate chronic venous insufficiency of the lower extremities.

6.2 Closest prior art

6.2.1 The board considers, in agreement with the decision under appeal and the parties, that the closest prior art is represented by the orally administered Antistax®, a commercially available product disclosed in D5, D6 and D36. Antistax® contains as active principle a mixture of an extract of red vine leaves (98,4%) and esculin (1,6%) and is also used for the treatment of venous insufficiency of the lower extremities (cf. D5, product 83 048; D6, abstract and pages 795-796, last section of the article; and D36 whole document).

Document D5 is silent about the composition of the red vine leaves extract used. In D6 and D36 it is stated that the main components of the extract are flavonoids (D6, page 793, right column, first full paragraph and D36 middle column under "1. Weinlaub-Extrakt") and that the properties of the flavonoids are significant for

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its use in the treatment of venous insufficiency (D6, page 794 section "*Venenleiden und Bioflavonoide*", in particular last paragraph and D36 middle column under "1. Weinlaub-Extrakt").

6.2.2 However, none of these documents disclose how the red vine leaves extract used in Antistax® is prepared. Thus, the composition of claim 1 differs from Antistax® as disclosed in these documents in that:

- it does not include esculin; and
- it is specified how the red vine leaves extract with a content of 2 to 20% flavonoids is prepared.

6.3 The problem to be solved and its solution

6.3.1 The compositions of claim 1 have the same use as the product Antistax®, namely the treatment of chronic venous insufficiency of the lower extremities. There is no evidence on file of any unexpected effect of the claimed compositions when compared with Antistax®. In fact, even the respondent saw the technical problem to be solved by the patent in suit in the provision of alternative compositions which are also useful for the treatment of venous insufficiency of the lower extremities.

6.3.2 This problem is solved by using the compositions defined in claim 1, wherein the active principle consists of an aqueous extract of red vine leaves containing 2 to 20% flavonoids and the extract is obtainable under specific extraction conditions.

In the light of the results of the study described in the patent specification the board is satisfied that

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the above-defined problem has been credibly solved. This finding was not challenged by the appellant.

6.4 Obviousness

6.4.1 It remains to be decided whether the suggested solution is obvious from the prior art. In this context it has to be analysed as to whether or not (i) the specific method of preparing the extract and (ii) the use of the red vine leaves extract alone can contribute to inventive step.

6.4.2 The respondent has maintained during the proceedings that the method of preparation of the red vine leaves extract results in an extract having very specific properties. It pointed out that plant extracts are complex mixtures defined by their method of preparation. The claimed extracts being characterized by a content of 2 to 20% flavonoids, mandatorily included 98 to 80% of other ingredients which were defined by the method of extraction. The respondent supported this affirmation by the two expert opinions, D34 and D35, indicating that two extracts were phytoequivalent, if their pharmaceutical equivalence and their therapeutical equivalence were demonstrated. As this evidence had not been provided by the appellant, it should be recognized that a different extract was used in the claims.

6.4.3 It is true that the method of preparation of the red vine leaves extract is not specified in the prior art, and thus a comparison of the claimed extracts with the extract used in Antistax® is not possible. In the absence of this information it has to be assumed, as

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pointed out by the appellant, that the prior art extract has been prepared according to the general knowledge of the skilled person using standard methods like the one disclosed in D4.

In D4 the extract is obtained by warm lixiviation of suitable, cut red vine leaves with water to complete exhaustion. The method used in D4 is described in a general way without indicating the specific conditions used in every process step.

Compared to this known process, the process of claim 1 requires:

(a) collecting red vine leaves at a point of time when the content in flavonoids has reached an optimum;
(b) drying and crushing the leaves;
(c) cutting the leaves to pieces; and
(d) extracting the leaves with water at temperatures from 60 to 80 °C for 6 to 10 hours in an exhaustive percolation.

Steps (a), (b) and (c) define in a very broad way how the leaves are handled before the extraction step. The leaves are to be collected when the content in flavonoids reaches an optimum and they are dried, crushed and cut into pieces. None of these steps are further concretized in the claim. In the absence of such further information it must be assumed that the skilled person would carry out these process steps according to the known standard technologies for the processing of medicinal plants (cf. D30 and D31) with the aim of optimizing the extraction of flavonoids, the known active components in red vine leaves. Steps (a),

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(b) and (c) thus cannot contribute to essentially distinguishing the claimed extract from the prior art extracts.

6.4.4 Concerning step (d), the actual extraction step, this step is carried out at a temperature in the range of 60° to 80°C for 6 to 10 hours in an exhaustive percolation to achieve a high content in flavonoids during the extraction as indicated in paragraph [0018] of the specification. This extraction step by percolation is similar to the lixiviation to complete exhaustion disclosed in D4 as both processes aim to extract the drug until exhaustion of the soluble substances.

In this context, the appellant provided further experimental evidence (D28) that the extraction conditions had little influence on the flavonoid content of a particular sample of red vine leaves. Thus, regardless of whether the temperature was inside or outside the range required in claim 1, whether percolation or maceration or even a mixture of water and ethanol was used, the content of flavonoids extracted from said sample, that is to say the known main active ingredients of the extract used in Antistax®, is always within the range of 2 to 20%.

Insofar as the respondent relied on the presence of compounds in the extract other than the main active ingredients, the board notes the following:

- The extract used in the patent in suit contains as main active ingredients quercetin-3-O-ß-D-glucuronide and isoquercitrin ([0009]). These are

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exactly the same main components present in the red vine leaves extract used in Antistax® according to D6 (page 793, right column, lines 25 - 29) and D36. The skilled person when preparing a red vine leaves extract for treating venous insufficiency would ensure that the components which are said to be the main active components are present in the extract in good yield.

– There is no information on file showing that the activity of the compositions could be due to other unspecified components of the extract which would not be extracted when carrying out the process of D4.

6.4.5 Thus, it is evident from the above that the extract obtained by steps (a) to (d) of claim 1 is very similar, if not identical, to the extract used in Antistax®. But even if there is no identity, the slight differences in the preparation leading to a slight difference in the extract, it has not been shown that slight variations in the preparation contribute to inventive step. On the contrary, all the process steps identified in claim 1 are common in this field.

The preparation of the red vine leaves extract can for these reasons not contribute to justifying an inventive step.

6.4.6 Concerning the use of the extract of red vine leaves alone, that is to say without esculin (question (ii) above), it is noted that D6 emphasizes the importance of the flavonoids present in the red vine leaves extract as the main active principle of the Antistax® product and being essentially responsible for its

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activity in the treatment of venous insufficiency (cf. D6, page 792, last sentence of the abstract and page 794, second and third columns). It would therefore be evident to the skilled person from studying these passages of D6 that a composition containing only the red vine leaves extract and being free of esculin would also show the desired activity.

This finding is also confirmed by further documents on file indicating that the red vine leaves extract was already known for the treating of venous insufficiency (for instance, D1, page 25) and that the effect on venous insufficiency is due to the presence of flavonoids (D36, middle column, "1. Weinlaub-Extrakt"). Furthermore, it is stated in D36, right hand column that esculin and the extract of wine leaves act in the same way ("gleichsinnig"). Nothing is said of the two components working in a synergistic way.

Consequently, the exclusion of esculin from the Antistax® product cannot justify the presence of an inventive step.

6.4.7 The respondent has filed document D37 during the appeal proceedings in order to show an unexpected result of the claimed compositions. In its opinion it was an unexpected result that the activity of the claimed composition would be maintained even if one active component of Antistax®, namely esculin, were removed. This was demonstrated when comparing the results of D37 with those of the patent in suit.

However no direct comparison of a treatment with the compositions of claim 1 with a treatment with Antistax®

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has been made. As discussed in detail during the oral proceedings it is rather difficult, if not impossible, to compare the results presented in the patent with the results of D37. The study in D37 was conducted with patients with only one leg suffering chronic venous insufficiency and its results cannot be compared with those of the patent conducted with patients suffering from chronic venous insufficiency in both legs. The assumption made by the respondent that the results in D37 were likely to be overestimated by a factor of 2 appears rather speculative. Consequently, no conclusion can be achieved by comparing both experimental results.

6.5 For these reasons the subject-matter of claim 1 of the main request lacks inventive step.

AUXILIARY REQUEST

7. *Inventive step (Article 56 EPC)*

7.1 Claim 1 of the auxiliary request is drafted in the form of a first medical use claim ("A dietary supplement composition for use in the prevention or alleviation of the discomfort associated with mild-to-moderate chronic venous insufficiency of the lower extremities …") which contains in addition to the features of claim 1 of the main request a dosage regime, namely that "the composition is administered in a daily dose of 300-800 mg of the extract in 1 to 3 capsules of tablets taken once daily" (point VIII above).

7.2 Since claim 1 of the main request already refers to "an active principle being capable of preventing or treating the discomfort associated with mild-to-

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moderate chronic venous insufficiency of the lower extremities", the mere reformulation of claim 1 to a first medical use claim cannot contribute to overcoming the inventive step objection against claim 1 of the main request.

Thus, the actual difference of the subject-matter of claim 1 of the auxiliary request over claim 1 of the main request is the dosage regime.

7.3 The recommended dose of Antistax® in D5 and D36 varies from 360 mg to 540 mg taken in two or three capsules twice a day. The now claimed dosage regime uses an amount of extract covering the known amount but it requires that it is taken only once a day.

7.3.1 It is well known that in designing dosage regimen the two major parameters that can be adjusted are the dose itself, i.e. the quantity of drug administered, and the dosing frequency, i.e. the time interval between doses.

7.3.2 In the present case the amount of active ingredient remains the same as the amount used in D5 and there is no evidence on file of any unexpected technical effect caused by administering the red vine leave extract once daily. The board considers that it is within the competence of the skilled person to find out the optimal dosage regime. Consequently, the replacement of a dosage regime by another dosage regime for the same purpose is considered to be a matter of routine experimentation and cannot be seen as involving an inventive step.

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7.3.3 In this respect the board cannot accept the argument of the appellant that it was unexpected that the same activity would be achieved by a change of dosage regime. In fact, a comparison of the claimed dosage regime with the dosage regime of D5/D36 has not been made. The only comparison which has been made compares the now required dosage regime with the use of compression stockings and/other oedema-reducing agents (last paragraph of the patent specification). The information given in the patent is that the red vine leave extract in a daily dose of 300-800 mg taken once daily is useful for treating chronic venous insufficiency. It has never been shown that taking a dose of 300-800 mg once daily has the same effect as taking the same dose twice daily.

Finally, insofar as the respondent relied on the fact that by changing the dosage regime from two times a day to only once a better adherence of the regime by patients is achieved, this advantage is in itself known and its exploitation in the present case cannot justify an inventive step.

7.4 Consequently, the subject-matter of claim 1 of the auxiliary request also lacks inventive step.

8. In summary, none of the requests relate to patentable subject-matter.

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Order

For these reasons it is decided that:

1. The decision under appeal is set aside.

2. The patent is revoked.

The Registrar The Chairman

D. Magliano W. Sieber

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Acknowledgement of receipt

We hereby acknowledge receipt of the Notice of Opposition:

Submission number	2041017
Application number	EP05712607.0
Patent number	EP1720571
Date of receipt	12 March 2013
Your reference	SR-01675
Opponent	Pfizer Inc.
Title	METHOD OF TREATING HEMOLYTIC DISEASE

Documents submitted

package-data.xml	ep-opposition-data.xml
ep-oppo.pdf (6 p.)	OTHER-1.pdf\SR-01675-Notice of oppo.pdf (1 p.)
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Correction by the EPO of errors in debit instructions filed by eOLF
Errors in debit instructions filed by eOLF that are caused by the editing of Form 1038E entries or the continued use of outdated software (all forms) may be corrected automatically by the EPO, leaving the payment date unchanged (see decision T 152/82, OJ EPO 1984, 301 and point 6.3 ff ADA, Supplement to OJ EPO 10/2007).

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Date
18-03-2013

Reference	Application No./Patent No.
M2367 EP	05712607.0 - 1412 / 1720571
Applicant/Proprietor	
ALEXION PHARMACEUTICALS, INC.	

Communication of a notice of opposition

You are hereby informed of a notice of opposition to the European patent specified above (see attached copy in the enclosure). The documents specified as patent documents in the notice of opposition are available for inspection via the European Patent Register at **www.epo.org/register**. Should you wish to receive these documents in paper format, they will be supplied to you free of charge if specifically requested on receipt of this communication (see OJ EPO 2009, 434).

If oral proceedings are to take place, parties are advised to check the electronic file via European Patent Register in advance of the hearing to ensure they are in possession of all relevant documents.

An invitation to file observations and to file amendments, where appropriate, to the description, claims and drawings (R. 79(1) EPC) will be issued separately.

The period within which such observations may be filed will not be fixed until the following conditions are met:

(a) the opposition period has expired;

(b) the notice of opposition has been examined for certain formal requirements (R. 77 EPC).

For the Opposition Division

Enclosure: Notice of opposition 01 with non-patent literature

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Office européen des brevets

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Pfizer
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Date
19-04-2013

Reference		Application No./Patent No.
SR-01675	OPPO 01	05712607.0 - 1412 / 1720571
Applicant/Proprietor		
ALEXION PHARMACEUTICALS, INC.		

Communication of further notices of opposition pursuant to Rule 79(2) EPC

No further opposition has been filed.

If oral proceedings are to take place, parties are advised to check the electronic file via the European Patent Register at **www.epo.org/register** in advance of the hearing to ensure that they are in possession of all relevant documents. (OJ EPO 2009, 434)

A copy of the communication pursuant to Rule 79(1) EPC sent to the proprietor of the patent is enclosed for your information.

The patent proprietor's (proprietors') observations on the notice of opposition to the above-mentioned patent will be communicated to the opponent without delay. A time limit for reply will be fixed if the Opposition Division considers this expedient.

If no reply to a communication is received within the time limit set, the proceedings will be resumed forthwith. Your attention is drawn to Article 114(2) EPC.

For the Opposition Division

Enclosures: copy of the communication pursuant to Rule 79(1) EPC (Form 2317A)

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Date
19-04-2013

Reference	Application No./Patent No.
M2367 EP	05712607.0 - 1412 / 1720571

Applicant/Proprietor
ALEXION PHARMACEUTICALS, INC.

Communication of notices of opposition (R. 79(1) EPC)

Notice of opposition has been filed within the opposition period by:

01. Pfizer Inc./235 East 42nd Street/New York, NY 10017/ETATS-UNIS D'AMERIQUE//

The notice of opposition indicated above has already been communicated to you.

You are requested to file your observations within a period of **four months** from notification of this communication.

You may also file amendments, where appropriate, to the description, claims and drawings within the period specified. One set of these documents is to be filed.

If you introduced documents which have not yet been mentioned during the proceedings, your attention is drawn to Rule 83 EPC.

Enclosures:

For the Opposition Division

Europäisches Patentamt • European Patent Office • Office européen des brevets •

Registered letter
EPO Form 2317A 12.07 (11/04/13) OBSO

VOSSIUS & PARTNER
Patentanwälte Rechtsanwälte

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European Patent Office

80298 M U N I C H

EPO - Munich
27
19. Aug. 2013

Opposition against EP-Patent 1720571
(Application No.: 05 71 2607.0)
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Our Ref.: M2367 OPP(EP)

München, August 19, 2013
KRD/FLG/OOV

Reference is made to the Communication dated April 19, 2013 in connection with the above-referenced case.

It is respectfully requested that the four-month period specified therein for filing observations be extended by two months, i.e. until

October 29, 2013.

As a precautionary measure oral proceedings are requested.

D. Krämer

Dana Krämer, Ph.D.
European Patent Attorney

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1) Japanese Patent Attorney
2) Fachanwalt für gewerblichen Rechtsschutz
3) U.S. Attorney-at-Law (New York)
4) European Patent Attorney
5) Registered U.S. Patent Attorney

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HR Firmennummer CH-270.9.001.271-9

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Date
26-08-2013

Reference	Application No./Patent No.
M2367 EP	05712607.0 - 1412 / 1720571

Applicant/Proprietor
ALEXION PHARMACEUTICALS, INC.

Extension of time limit pursuant to Rule 132 EPC

Opposition procedure

With reference to your request, the time limit for replying to the communication dated 19.04.2013 has been extended

by 2 months

to a total of 6 months

from the date of notification of the above-mentioned communication.

Please note: To the extent that your request exceeded the above extension, your request has been refused.

Note:
The granting of extensions to time limits is governed by the Implementing Regulations to the EPC and the Guidelines for Examination in the EPO, part E-VII, 1.6. A request for extension, which would result in the total period set exceeding six months, must be reasoned and supported by evidence.

If no reply to the communication is received in due time, the procedure will be continued. Attention is drawn to Article 114(2) EPC.

For the Opposition Division

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Date
26-08-2013

Reference	Application No./Patent No.
SR-01675 OPPO 01	05712607.0 - 1412 / 1720571
Applicant/Proprietor: ALEXION PHARMACEUTICALS, INC.	

BRIEF COMMUNICATION

Subject:
- ☐ Your letter of
- ☐ Our telephone conversation of
- ☐ Communication of
- ☐

Enclosure(s):
- ☒ Letter from the proprietor of the patent of 19.08.2013
- ☐ Letter from the opponent of
- ☒ Copy (copies) EPO Form 2944C

Communication:

☐ A letter from the opponent /proprietor was received on ..... . The documents specified as patent documents in this letter are available via the European Patent Register at **www.epo.org/register.** Should you wish to receive these documents in paper format, they will be supplied to you free of charge if specifically requested on receipt of this communication (OJ EPO 2009, 434).

☐

For the Opposition Division

Europäisches Patentamt • European Patent Office • Office européen des brevets

☐ **Registered letter**

EPO Form 2911O 01.12 (21/08/13)

AG21431

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Direct Fax: +33 1 70 70 72 37
Email: ~IPGSParis@pfizer.com

European Patent Department

VIA EPOLINE

EUROPEAN PATENT OFFICE
Erhardtstrasse 27
D-80298 Munich
Germany

07 October, 2013

OUR REF.: SR-01675
THP/IC

Re: European Patent No 1 720 571 B1 (ALEXION PHARMACEUTICALS, INC.)
Application No. 05712607.0
Opponent: PFIZER INC.

Dear Sirs,

The opponent, Pfizer, Inc., hereby formally and irrevocably withdraws its opposition to EP1720571B1 in the name of Alexion Pharmaceuticals, Inc. entitled "Method of treating hemolytic disease".

Yours faithfully,

Thomas Pringot
European Patent Attorney
Association Pfizer - No. 462
Phone: +33-1 58 07 43 69
Fax: +33-1 70 70 72 37
Email: thomas.pringot@pfizer.com

Europäisches Patentamt
European Patent Office
Office européen des brevets

Submission in opposition proceedings

Representative:

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- representing the opponent(s):	Pfizer Inc.
Opponent/representative's reference	SR-01675

The information given below is pertaining to the following patent in opposition proceedings:

Patent No.	EP1720571
Application No.	EP05712607.0
Date of mention of the grant in the European Patent Bulletin (Art. 97(3), Art. 99(1) EPC)	13 June 2012
Title of the invention	METHOD OF TREATING HEMOLYTIC DISEASE
Proprietor of the patent	ALEXION PHARMACEUTICALS, Inc.

Requests:

- Opposition is herewith withdrawn.

Documents attached:

	Description of document	Original file name	Assigned file name
1	Any annexes (other than citation) to an opposition letter - Withdrawal of opposition	SR-01675-Withdrawal.pdf	OTHER-1.pdf

Signatures

Place:	**Paris**
Date:	**07 October 2013**
Signed by:	**/Thomas PRINGOT/**
Association:	**462**
Capacity:	**(Representative)**

Samsung Bioepis Exhibit No. 1027
Page 261

Europäisches Patentamt
European Patent Office
Office européen des brevets

Acknowledgement of receipt

We hereby acknowledge receipt of the following submission by the opponent:

Submission number	2336621
Application number	EP05712607.0
Patent number	EP1720571
Date of receipt	07 October 2013
Your reference	SR-01675
Opponent	Pfizer Inc.
Title	METHOD OF TREATING HEMOLYTIC DISEASE
Documents submitted	package-data.xml ep-oppo.pdf (2 p.) ep-opposition-data.xml OTHER-1.pdf\SR-01675-Withdrawal.pdf (1 p.)
Submitted by	CN=Isabelle Campion 12135
Method of submission	Online
Date and time receipt generated	07 October 2013, 16:17 (CEST)
Message Digest	62:F8:CB:C6:AD:87:27:49:C0:A7:F1:61:7A:40:34:8F:17:4E:97:6C

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

/European Patent Office/

VOSSIUS & PARTNER

Patentanwälte Rechtsanwälte

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

European Patent Office

80298 MUNICH

EPO - Munich
81
09. Okt. 2013

05 71 2607.0 / 1720571
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Our Ref.: M2367 OPP(EP)

München, October 9, 2013
WA/FLG

This is in reply to the Communication of notices of opposition (Rule 79(1) EPC) issued in the above-referenced opposition proceedings on April 19, 2013.

The Opponent, Pfizer Inc., formally and irrevocably has withdrawn its opposition on October 7, 2013. It is therefore respectfully requested that the patent is maintained as granted and the opposition proceedings are promptly terminated.

Dr. Joachim Wachenfeld
European Patent Attorney

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4) European Patent Attorney
5) Registered U.S. Patent Attorney

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Date: 09-10-2013

Reference	Application No./Patent No.
SR-01675 OPPO 01	05712607.0 - 1412 / 1720571
Applicant/Proprietor: ALEXION PHARMACEUTICALS, INC.	

BRIEF COMMUNICATION

Subject:
- ☒ Your letter of 07.10.13
- ☐ Our telephone conversation of
- ☐ Communication of
- ☐

Enclosure(s):
- ☐ Letter from the proprietor of the patent of
- ☐ Letter from the opponent of
- ☐ Copy (copies)

Communication: Your above notice of withdrawal of opposition is hereby acknowledged. You are no more a party in this proceedings.

☐ A letter from the opponent /proprietor was received on ..... . The documents specified as patent documents in this letter are available via the European Patent Register at **www.epo.org/register.** Should you wish to receive these documents in paper format, they will be supplied to you free of charge if specifically requested on receipt of this communication (OJ EPO 2009, 434).

☐

For the Opposition Division

Europäisches Patentamt • European Patent Office • Office européen des brevets

☐ **Registered letter**

EPO Form 2911O 01.12 (09/10/13)

CC20871

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Date
09-10-2013

Reference	Application No./Patent No.
M2367 EP	05712607.0 - 1412 / 1720571
Applicant/Proprietor	
ALEXION PHARMACEUTICALS, INC.	

BRIEF COMMUNICATION

Subject:
- ☐ Your letter of
- ☐ Our telephone conversation of
- ☐ Communication of
- ☐

Enclosure(s):
- ☐ Letter from the proprietor of the patent of
- ☒ Letter from the opponent of 07.10.13
- ☒ Copy (copies) of brief comm. of 09.10.13

Communication:

☐ A letter from the opponent /proprietor was received on ..... . The documents specified as patent documents in this letter are available via the European Patent Register at **www.epo.org/register.** Should you wish to receive these documents in paper format, they will be supplied to you free of charge if specifically requested on receipt of this communication (OJ EPO 2009, 434).

☐

For the Opposition Division

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☐ **Registered letter**

EPO Form 2911O 01.12 (09/10/13)

CC20871

VOSSIUS & PARTNER
Patentanwälte Rechtsanwälte

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

European Patent Office

80298 M U N I C H

EPO - Munich
44
29. Okt. 2013

Opposition against EP 1720571
(Application No.: 05 71 2607.0)
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Our Ref.: M2367 OPP(EP)

München, October 29, 2013
WA/ARO/BUK

Reference is made to the Communication dated April 19, 2013 as well as our petition of October 9, 2013 in the above-mentioned case.

In view of the recent withdrawal of the opposition by Pfizer Inc. and the fact that so far we have not heard back from the Opposition Division, we would respectfully ask for an exceptional two-month extension of time, i.e. until

December 29, 2013.

Only in case the Opposition Division would be inclined to continue the proceedings and our above request for extension would not be granted, we request that nevertheless we get a further opportunity to file observations in written form.

As a precautionary measure oral proceedings are requested.

Dr. Joachim Wachenfeld
European Patent Attorney

PATENTANWÄLTE - DEUTSCHLAND
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Reference	Application No./Patent No.
M2367 EP	05712607.0 - 1412 / 1720571
Applicant/Proprietor	
ALEXION PHARMACEUTICALS, INC.	

Extension of time limit pursuant to Rule 132 EPC

Opposition procedure

With reference to your request, the time limit for replying to the communication dated 19.04.2013 has been extended

by 2 months

to a total of 8 months

from the date of notification of the above-mentioned communication.

Please note: To the extent that your request exceeded the above extension, your request has been refused.

Note:
The granting of extensions to time limits is governed by the Implementing Regulations to the EPC and the Guidelines for Examination in the EPO, part E-VII, 1.6. A request for extension, which would result in the total period set exceeding six months, must be reasoned and supported by evidence.

If no reply to the communication is received in due time, the procedure will be continued. Attention is drawn to Article 114(2) EPC.

For the Opposition Division

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Patent No.: EP-B-1 720 571

Preparation for oral proceedings - Instructions to Support Service

Oral proceedings are to be held in connection with the above patent application

1. The matters to be discussed are set out in the annex (Form 2906)

2. Dispatch the summons using Form 2008/2310 and Form 2906 for the parties to attend on:

Day 15.05.2014 Time 09:15

ROOMS

09.01.14 CI
1662
Room booked

ORAL 01, 02, 03 and 05 coded

Date 09.01.14 Initials

2.1 If no room is available, notify the division on Form 2088

2.2 Parties' submissions in preparation for the oral proceedings, if any, should be made no later than

1 month(s)

before the date of the oral proceedings
(transfer to Form 2008.1 / 2310.1)

2.3 Encode ORAL(04) coded 09.01.14 CI

Date Initials

2.4 Dispatch Form 2008.7 / 2310.7 to division

Date Initials

3. ☐ Arrange for the following special equipment to be provided in the conference room:

Date Initials

4. Request language service to provide simultaneous interpretation facilities as necessary

Date Initials

5. Return the dossier to primary examiner with Form 2041 (15 days before the oral proceedings)

Date Initials

6. Check that summons has been received (Form 2936 / advice of delivery)

7. 15 days before the oral proceedings:
- dispatch the dossier to the primary examiner and
- dispatch Form 2041 with copies for the other members of the examining division.

19.12.2013
Date

Cilensek, Zoran	Weikl, Martina	Chapman, Rob	
Chairman	2nd examiner	1st examiner	Legal member

Enclosure(s):

Opposition against EP 1720571
Patentee: Alexion Pharmaceuticals, Inc.
Our Ref.: M2367 OPP(EP)

1017

D13

Eculizumab Alexion

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Eculizumab (5G1.1), a humanized monoclonal antibody that prevents the cleavage of human complement component C5 into its pro-inflammatory components, is under development by Alexion as a potential treatment for several chronic inflammatory diseases, including rheumatoid arthritis (RA) and nephritis [190673], [292328]. In January 2002, a phase IIb trial was initiated for RA [437814]. This trial was ongoing in April 2002, at which time, eculizumab was also in phase II trials for the treatment of membranous nephritis and lupus nephritis, and in earlier stage clinical trials for dermatomyositis and pemphigoid [446377]. The company is also developing a single-chain version of this antibody, pexelizumab, for use in acute cardiovascular indications [188760].

In October 2000, eculizumab was granted Orphan Drug status by the FDA for the treatment of dermatomyositis [385057]. In February 2002, the product received Orphan Drug designation for its use in patients with membranous nephritis [440583].

In September 2001, analysts at US Bancorp Piper Jaffray predicted eculizumab's launch for dermatomyositis and pemphigus in 2003, RA and nephritis in 2004, and chronic heart failure (CHF) after 2006 [426537]. In March 2002, analysts at US Bancorp Piper Jaffray predicted that the product would have peak worldwide sales in RA of US $175 million and US $400 million for nephritis. Sales for the RA indication are predicted to reach US $35 million in 2006, rising to US $110 million in 2008, and US $10 million in 2006, rising to US $50 million in 2008, in the US and the rest of the world, respectively. For the nephritis/other indication(s), sales are pegged at US $50 million in 2006, rising to US $200 million in 2008, and US $15 million in 2006, rising to US $100 million in 2008, again in the US and the rest of the world, respectively [446992].

Introduction

The complement system comprises more than 30 human proteins in plasma and on cell surfaces that interact in a cascade sequence to mediate a variety of inflammatory events. These events include the opsonization of particles for phagocytosis, leukocyte activation and assembly of the membrane attack complex (MAC) [435653], [435654], [435718], [445228]. The system is responsible for killing and removing organisms, cells and cell components from the circulation which are recognized as foreign. In addition to host defense against bacterial agents, complement is involved in the disposal of immune complexes and the products of inflammatory injury, and serves as an interface between innate and adaptive immunity [435653].

Originator Alexion Pharmaceuticals Inc

Status Phase II Clinical

Indication Dermatological disease, Glomerulonephritis, Nephritis, Pemphigus, Psoriasis, Rheumatoid arthritis, Systemic lupus erythematosus

Action Complement cascade inhibitor

Biotechnology Monoclonal antibody, humanized

Synonyms 5G1.1, h5G1.1, C5 complement inhibitor (Alexion), h5G1.1scFv

CAS 5G1.1
Registry No: 339087-76-2

Several complement proteins are cleaved during activation of the complement system and three pathways of activation have been described; the classical, the alternative and the lectin pathways [435653]. The pathways leading to the cleavage of C3 are triggered enzyme cascades. Downstream in these pathways, C3 is cleaved into C3a and C3b. C3a is released while C3b forms C5 convertases, which in turn cleave C5 to C5a and C5b. Following complement activation, pro-inflammatory peptides, such as the anaphylotoxins C3a and C5a, are generated and C5b-9 (MAC) is formed. Assembly of the MAC from the terminal components (C5 to C9) of the cascade leads to membrane damage. In addition, complement activation products, especially the anaphylotoxins, elicit a number of biological effects, including chemotaxis of leukocytes, degranulation of phagocytic cells, mast cells and basophils, smooth muscle contraction and the increase of vascular permeability [435653], [445228], [445736].

Under normal circumstances, the regulatory mechanisms of complement are balanced, so that the activation of complement occurs on the surface of invading microorganisms and the deposition of complement on normal cells and tissues is usually limited [435653], [445228]. If the mechanisms that regulate this balance are abnormal, the complement system might then cause tissue injury [435656]. Excessive or inappropriate activation of the complement system can lead to harmful and life-threatening consequences which are secondary to severe inflammatory tissue destruction. These consequences can manifest clinically in a variety of ways, including multiple organ failure, hyperacute graft rejection and septic shock [435656]. Inappropriate complement activation and its deposition on host cells can also lead to direct complement-mediated cell lysis of target structures, or indirectly to tissue destruction due to the generation of powerful mediators of inflammation [435653], [445228].

Complement plays an important role in the pathogenesis of many autoimmune and inflammatory diseases, including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) [435694], [445236] and dermatomyositis [445242]. It is also responsible for disease states associated

with bio-incompatibility, including transplant rejection [445249]. In addition, complement activation plays an important role in the pathogenesis of cardiac and intestinal ischemia/reperfusion injury [445251]. Other conditions in which complement activation has been proposed to play a role include stroke [445254], multiple sclerosis [445384], pancreatitis [445255], psoriasis [445272], Alzheimer's disease [445278] and prion-induced diseases [445286].

Therapeutic inhibition of complement is, therefore, potentially important in protecting against the development of certain diseases and it has been shown to be useful in animal models of sepsis, intestinal ischemia/reperfusion injury, myocardial reperfusion injury, nephritis, arthritis and graft rejection.

Several complement inhibitors are currently under investigation [212463], [225318], [281110], [435656], [435722], [435723], [435725], [436916]. Eculizumab (5G1.1), under development by Alexion Pharmaceuticals Inc, is a humanized C5 inhibitory monoclonal antibody (mAb) that prevents the cleavage of human complement component C5 into its pro-inflammatory components and targets the complement components, C5a and C5b-9 [445382]. As mentioned, the early steps of the complement cascade play an important role in the removal of infectious agents and several normal functions of the immune system. The rationale of specifically blocking C5 is that the normal upstream disease-preventing functions of complement remain intact, while the production of the abnormal downstream disease-causing actions of complement are blocked. This antibody, therefore, potentially avoids the disruption of antibacterial-protective mechanisms, which are believed to be mediated primarily through C3b [438165].

Eculizumab is undergoing phase II clinical trials for a variety of chronic inflammatory conditions, and is being developed as a potential treatment for RA, SLE and nephritis [190673], [292328], [412090]. The drug is currently in phase II trials for nephritis and has completed phase I/II trials in SLE and phase II trials in RA [328001], [335241], [335995], [396672]. Eculizumab is also currently undergoing phase I trials for psoriasis and pemphigus [384948], and has been granted Orphan Drug status by the FDA for the treatment of dermatomyositis [385057], and membranous nephritis [440583].

Alexion has also developed a short-acting C5 complement inhibitor that is a humanized single chain mAb fragment. This compound is known as pexelizumab (formerly 5G1.1-SC) and it is being tested for the treatment of acute cardiovascular diseases associated with complement activation, including acute myocardial infarction (MI), unstable angina and revascularization/reperfusion therapies. This drug was designed to have the advantage of enhanced tissue penetration, desirable for effective treatment of these acute life-threatening conditions [188760]. Pexelizumab and eculizumab are currently in at least eight clinical development programs [435963]. A phase III clinical study with pexelizumab in cardiopulmonary bypass patients has been initiated. In addition, two large phase II studies with pexelizumab in acute myocardial infarction (MI) patients are under way. Since pexelizumab has recently been evaluated, this review will focus primarily on eculizumab.

Synthesis and SAR

Humanized 5G1.1, Fab and scFv molecules were produced by grafting the complementary determining regions of 5G1.1 on to human framework regions [258884]. Competitive ELISA analysis indicated that no framework changes were required in the humanized variable regions for retention of high affinity binding to C5, even at framework positions predicted by computer modeling to influence CDR canonical structure. The humanized Fab and scFv molecules blocked complement-mediated lysis of chicken erythrocytes and porcine aortic endothelial cells in a dose-dependent fashion. Complete complement inhibition occurred at 3-fold molar excess, relative to the human C5 concentration. Humanized 5G1.1 scFv also effectively blocked C5a generation. In addition, intact humanized 5G1.1 antibody blocked human complement lytic activity at concentrations identical to the original murine monoclonal antibody, indicating that humanized 5G1.1 and its derivatives retained both the affinity and blocking functions of murine 5G1.1 antibody [258883], [258884]. 5G1.1 is supplied as a sterile, non-pyrogenic solution (2 mg/ml) for iv injection [212467], [258884] and it has picomolar affinity for a sequence within human C5. 5G1.1 can be administered either intramuscularly or subcutaneously, allowing self-administration [438165].

Pharmacology

Heart disease

There is a growing body of evidence pointing to a substantial disease-promoting role of complement components in MI and unstable angina [445289]. Activation of the complement cascade in MI patients is shown by elevated levels of activated complement byproducts in the blood of patients during acute MI and by the deposition of activated complement components in damaged heart tissue [445382]. Interestingly, terminal complement components play a key role in the apoptotic process in heart tissue during MI [241313], and blockade of the complement cascade at C5 has been shown to substantially reduce myocardial apoptosis and tissue damage in rats [241313]. Complement activation was also found in patients undergoing cardiopulmonary bypass (CBP) [445311]. This finding was associated with increased patient morbidity, including MI, stroke, cognitive dysfunction and blood loss [445311].

Studies in primates demonstrated that inhibition of complement activation reduces heart damage associated with coronary ischemia and reperfusion in preclinical models. Furthermore, treatment of hypoxic/reoxygenated human umbilical vein endothelial cells (HUVECs) with h5G1.1 scFv attenuated C5b-9 deposition, NFκB translocation and vascular cell adhesion molecule (VCAM)-1 expression [353137].

Intestinal and lung ischemia-reperfusion

A recent study reported that anti-C5a therapy significantly improved intestinal ischemia-reperfusion tissue injury as well as lung injury in rats [445313]. These results suggested that C5 inhibition could also limit the complaints in the broader general surgery population as well as in patients with ulcerative colitis [357827] Interestingly, C5 inhibition was associated with a significant reduction in tumor necrosis factor (TNF)α levels [357827].

Inflammatory arthritis

Animals deficient in the complement component C5 are not susceptible to the onset of active arthritis [363495]. In the absence of C5 cleavage, the presence of TNFα is not sufficient to promote arthritis. A recent study reported that administration of anti-C5 mAb ameliorates the established collagen-induced arthritis in mice and rats [190673], [212456]. Systemic administration of the anti-C5 mAb effectively inhibited terminal complement activation *in vivo* and prevented the onset of arthritis in immunized animals. In addition, anti-C5 mAb treatment was also highly effective in ameliorating established disease. These results suggested a critical role for activated terminal complement components, not only in the induction, but also in the progression of collagen-induced arthritis [190673], [212456].

Renal disease and lupus

A C5 inhibitor was administered in both an acute and rapid progressive glomerulonephritis (RPGN) model that normally leads to crescentic changes, the most common histological lesion observed in lupus nephritis. The inhibitor was also administered to the NZB/NZW F1 lupus-prone mouse model. C5 inhibition prevented histological and clinical development of nephritis in the RPGN model and C5 inhibitor administration in the lupus-prone mouse model reduced histologic and biochemical evidence of kidney disease. Survival at 10 months was markedly improved from 5% in placebo-treated lupus-prone mice to 85% in C5 inhibitor-treated lupus-prone mice [190673], [232606], [280073].

Hematological diseases

C5 inhibition has also been shown to prevent platelet loss in a model of idiopathic thrombocytopenic purpura (ITP) [392090].

Metabolism

5G1.1 blocks complement activity for 1 to 2 weeks after a single dose [438165].

Toxicity

No toxicity has been reported in humans [228105], [392382] or rhesus monkeys [212462]. Interestingly, a recent study reported that C5 plays an important role in liver regeneration, strongly implicating the complement system as a crucial regulatory component of hepatic homeostasis. C5-deficient mice show severely defective liver regeneration and persistent parenchymal necrosis after exposure to CC_{l_4}. In addition, these mice show marked delays in the re-entry of hepatocytes into the cell cycle (S phase) and diminished mitotic activity. Reconstitution of C5-deficient mice with murine C5 or C5a significantly restores hepatocyte regeneration after toxic injury. Furthermore, blockade of the C5a receptor abrogates the ability of hepatocytes to proliferate in response to liver injury [445317]. The role of eculizumab on hepatic regeneration is unclear at this point.

C5 is also known to mediate chemotactic and activation events that are the basis for granulomatous responses during murine tuberculosis. Mycobacterium tuberculosis-infected mice with natural deficiency in C5 are unable to develop productive granulomatous responses, and are impaired in limiting organism growth within the lung [445321].

Clinical Development

Phase I

Rheumatoid arthritis

A phase I/II, multicenter, double-blind, placebo-controlled, dose-escalating trial tested the role of eculizumab in ameliorating RA. The trial was designed to gather clinical data on the safety profile and biological effects of a single administration of eculizumab in RA. The preliminary analysis demonstrated that the drug was well tolerated and was associated with a significant reduction in disease activity in these patients [445382]. The safety and biological activity of the antibody was examined in 40 patients with mild-to-moderate RA, each of whom received a single dose of eculizumab (0.1 to 8 mg/kg). The drug was safe and well tolerated, and showed no detectable immunogenicity in the population studied. Furthermore, a single dose of eculizumab potently and rapidly blocked complement activity in a dose-dependent fashion for up to 2 weeks [353141]. Complement inhibition after a single dose was associated with a beneficial clinical effect, with the highest dose (8 mg/kg; n = 6) resulting in a significant 30% reduction in C-reactive protein levels, compared to placebo-treated patients in which C-reactive protein increased by 24%. Eculizumab was also associated with a trend in reductions in other measurement of disease activity, including tender joint count, swollen joint count, patients' global assessment of disease and patients' global assessment of pain [291142], [305950], [322159], [335241], [347452], [335995].

Systemic lupus erythematosus

Alexion has completed preliminary analysis of the phase I study of eculizumab in SLE. The trial was designed to gather data on pharmacodynamics, safety and biological effects of a single administration of the drug in SLE patients. Eculizumab was well tolerated and its administration was associated with significant reduction in the incidence of proteinuria. The phase I, double-blind, placebo-controlled, dose-escalating trial examined the safety and biological activity of eculizumab in 24 patients with mild SLE, each of whom received a single dose of the antibody (0.1 to 8 mg/kg). There was no detectable immunogenicity in the patients studied and a single dose of antibody blocked complement activity in patients for up to 2 weeks. Administration of a single dose of 8 mg/kg was associated with significant decrease in proteinuria [292328], [328001].

Dermatomyositis

In 2001, Alexion completed a 2-month, phase I, pilot safety trial of eculizumab in 13 dermatomyositis patients with persistent dermatomyositis, undergoing concomitant treatment with moderate doses of methotrexate or steroids. The patients were evaluated in either a placebo (n = 3) or a single drug treatment arm (n = 10). Drug treatment consisted of eculizumab (8 mg/kg/week iv) for 5 weeks and then 8 mg/kg every 2 weeks for up to 2 months. The patients were evaluated after 2 months of treatment for safety and for trends in clinical improvement. In this placebo-controlled, multicenter trial, the drug was safe and well tolerated and associated with an improvement in skin rash. Exploratory clinical measurements included clinical and laboratory assessments of skin rash and muscle strength. There were consistent trends in improvements with drug administration in subjective and objective

measurements of skin rash during the 2-month trial. While there was little baseline skin inflammation in the placebo group, a majority of drug-treated patients who completed the trial experienced an improvement of ≥ 50% in their skin rash score [354030], [434351], [435888].

Pemphigoid

Published observations have shown that clinical improvement of pemphigoid is associated with reduced levels of complement activation in the previously affected skin, further supporting the rationale for testing the C5 inhibitor in this disease. Phase I trials of eculizumab in pemphigoid syndrome are ongoing [361798], [426537], [435888], but no data are currently available.

Psoriasis

A single-center, double-blind, placebo-controlled study was designed to evaluate the safety profile and clinical effects of eculizumab in severe psoriasis [352110]. In June 2001, Alexion completed a phase I pilot safety trial involving 40 psoriasis patients, which showed that the drug was well tolerated. Drug administration did not influence the clinical outcome as measured by Psoriasis Area and Severity Index (PASI) score, although favorable trends in certain measures of disease activity were observed. Drug administration dose-dependently blocked hemolytic activity in the blood of treated patients and dose-dependently reduced deposition of activated terminal complement in psoriatic plaques [412091].

Phase II

Rheumatoid arthritis

In a phase II trial in 209 patients, eculizumab administration appeared to be safe and well tolerated and the adverse event profile was comparable to placebo. Patients were treated with placebo, eculizumab (8 mg/kg iv) once per week for 4 weeks and then once every month (induction/monthly group); eculizumab (8 mg/kg iv) once per week for 4 weeks followed by once every 2 weeks (induction/biweekly group); or, eculizumab (8 mg/kg iv) once every 2 weeks (biweekly group). The results after 3 months of treatment showed that the induction/monthly group met the primary endpoint of the trial (improvement in American College of Rheumatology response criteria score (ACR) 20 after 3 months of treatment), while the induction/biweekly and biweekly groups did not statistically meet the endpoint. The ACR20 response in the induction/monthly group was 44% as compared to 18% ACR20 in the placebo group, by per protocol analysis. Both induction/monthly and induction/biweekly groups also met the secondary endpoint of changes in C-reactive protein after 3 months of therapy [429348].

In patients with elevated C5b-9 levels over 200 ng/ml, the ACR20 at 3 months of treatment were dose-dependent. The ACR20 results obtained in patients with elevated baseline C5b-9 levels were: placebo (9%); biweekly (33%); induction/monthly (57%); and induction/biweekly (50%). Further data from this trial are to be released following unblinding [397163], [429348].

Alexion has also started a phase IIb trial in RA patients. The trial is designed to assess the safety and efficacy of eculizumab and to confirm the most efficacious dose regimen. The trial will consist of approximately 300 patients with mild-to-moderate disease who are being treated concomitantly with disease-modifying antirheumatic drugs such as methotrexate or leflunomide. The trial will consist of three treatment arms; patients will be treated with placebo; eculizumab (8 mg/kg/week iv) for 4 weeks and then once every month; or eculizumab (8 mg/kg/week) for 4 weeks and then bimonthly. The patients will be evaluated after a 6-month drug phase for safety and efficacy, and the primary efficacy endpoint will be the ACR20 score [437814].

Systemic lupus erythematosus and nephritis

In August 1999, Alexion initiated a phase II study with eculizumab in lupus patients suffering from membranous nephritis [335995]. The trial is expected to enroll approximately 40 lupus nephritis patients at four clinical sites in the US and has been designed to test the safety and biological efficacy of chronic administration of eculizumab for up to 6 months [412090]. In September 2001, Alexion expected phase II results to be available in mid-2002 [426537].

Side Effects and Contraindications

In clinical trials to date, the drug was well tolerated and showed no detectable immunogenicity in the patients studied. No major adverse reactions have been reported. In the dermatomyositis trial, the most common side effects were headache and rash, and it appeared that these side effects were comparable to the placebo group. In the psoriasis trial, the most common side effects were headaches and unspecified pain. The most common side effects in the RA trial were comparable to placebo and included diarrhea and headaches [429348], [434351].

Patent Commentary

In March 2002, Alexion was issued US-06355245 entitled 'C5-specific antibodies for the treatment of inflammatory diseases.' The patent covers the composition of Alexion's lead drug candidates, eculizumab and pexelizumab, and other C5-binding anti-inflammatory antibodies [443717]. WO-09529697, entitled 'methods and compositions for the treatment of glomerulonephritis and other inflammatory diseases' describes a method for the possible treatment of glomerular inflammation and enlargement, involving the administration of low dosages of anti-C5 antibodies. WO-09609043 describing a method for the treatment of established joint inflammation is claimed in which a C5 blocker is administered.

Current Opinion

C5 inhibitors appear to intervene at a point that allows preservation of the anti-inflammatory and antibacterial responses at the C3 level, while conceivably inhibiting the downstream disease-causing actions. Selective suppression of this immune response could certainly provide a significant therapeutic advantage compared to existing therapies. Eculizumab blocks the production of harmful complement components and appears to be promising for the treatment of various inflammatory diseases, including RA, SLE and a variety of conditions with skin involvement, including dermatomyositis, pemphigoid and psoriasis. There are other conditions in which C5a seems to play an important role, including asthma and HIV infection, and these conditions might potentially become therapeutic targets in which C5a inhibition could be useful [445327], [445336].

Since complement activation has both positive and negative effects, the risk associated with complement inhibition is probably not negligible and long term studies will be necessary to assess possible risks. In particular, a theoretical concern is the role that C5 plays in hepatic regeneration and in combating granulomatous infections such as tuberculosis. Long term studies will determine whether C5 inhibition could have deleterious effects in these processes.

So far, limited animal and human studies have shown that eculizumab inhibits complement in a dose-dependent manner, is well tolerated and has no significant side effects. The results have been encouraging so far but more clinical trials are needed. There are still concerns that this drug does not block activation of the early stages of the complement system. This inhibitor allows the generation of C3a. While C3a is considered a less potent inflammatory molecule than C5a, the C3a receptor tissue expression appears to be much broader than originally expected [445349], [445352], and recent studies have shown that C3a can induce the production of inflammatory cytokines in a fashion similar to C5a [445354]. C3a could, in theory, play a significant role in inflammatory conditions and acute-phase response even after C5 inhibition. Nevertheless, if clinical trials prove successful, this drug as well as pexelizumab, could have a broad range of applications where complement-mediated inflammation contributes to disease pathology. There are currently no biological therapies on the market with the actions and specificity of these drugs and eculizumab could have a very strong therapeutic potential.

Development history

Developer	Country	Status	Indication	Date	Reference
Alexion Pharmaceuticals Inc	US	Phase II clinical	Rheumatoid arthritis	09-JUL-98	291142
Alexion Pharmaceuticals Inc	US	Phase II clinical	Nephritis	12-AUG-99	335995
Alexion Pharmaceuticals Inc	US	Phase I clinical	Psoriasis	06-OCT-00	384948
Alexion Pharmaceuticals Inc	US	Phase I clinical	Dermatological disease	06-OCT-00	384948
Alexion Pharmaceuticals Inc	US	Phase I clinical	Pemphigus	06-OCT-00	384948
Alexion Pharmaceuticals Inc	US	Preclinical	Glomerulonephritis	29-JUL-97	190673
Alexion Pharmaceuticals Inc	US	NDR	Systemic lupus erythematosus	11-APR-02	-

Literature classifications

Biology

Study Type	Effect Studied	Experimental Model	Result	Reference
In vivo	Anti-arthritic effect.	Collagen-induced arthritis in mice and rats.	An anti-C5 antibody both prevented the onset of disease and reduced established disease.	190673 212456
In vivo	Reduction in evidence of kidney disease.	NZB/NZW F1 lupus-prone mouse model.	An anti-C5 antibody reduced both the histological and biochemical signs of nephritis, and increased survival to 85% at 10 months.	232606

Metabolism

Effect Studied	Model Used	Result	Reference
Pharmacokinetics.	Complement activity.	A single dose of eculizumab blocks complement activity for 1 to 2 weeks.	438165

Clinical

Effect Studied	Model Used	Result	Reference
Safety and efficacy.	Phase I trial in 40 psoriasis patients.	Eculizumab was well tolerated and dose-dependently reduced complement activity in patients. Favorable trends were observed in PASI, although these were not significant.	412091
Safety and efficacy.	Phase I trial in 13 patients with persistent dermatomyositis.	Eculizumab (8 mg/kg/week iv) for 5 weeks, then every 2 weeks for up to 2 months, was safe and well tolerated. Drug treatment reduced skin rash score by ≥ 50%.	434351
Safety and efficacy.	Double-blind, placebo-controlled, phase I, dose-escalating trial in 24 patients with mild SLE.	Eculizumab (0.1 to 8 mg/kg) was safe and well tolerated, and no detectable immunogenicity was observed. The highest dose significantly reduced proteinuria.	328001
Safety and efficacy.	Phase II trial in 209 RA patients.	Eculizumab produced ACR20 in 44% of the patients treated. Adverse effects were similar to placebo, with nausea and diarrhea being the most common complaints.	429348
Safety and efficacy.	Phase II trial in RA patients in whom the C5b-9 levels were > 200 ng/ml.	A total of 57% of patients achieved ACR20 when treated with induction/monthly treatment of eculizumab.	429348

Associated patent

Title Method for reducing immune and hemostatic dysfunctions during extracorporeal circulation.

Assignee Alexion Pharm Inc

Publication WO-09525540 28-SEP-95

Priority US-00217391 23-MAR-94

Inventors Rollins SA, Smith BR, Squinto SP.

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L27 ANSWER 2 OF 2 REGISTRY COPYRIGHT 2009 ACS on STN
RN 219685-50-4 REGISTRY REGISTRY COPYRIGHT 2009 ACS on STN
RN 219685-50-4 REGISTRY
ED Entered STN: 14 Feb 1999
CN Immunoglobulin, anti-(human complement C5 .alpha.-chain) (human-mouse monoclonal 5G1.1 heavy chain), disulfide with human-mouse monoclonal 5G1.1 light chain, dimer (9CI) (CA INDEX NAME)
OTHER NAMES:
CN Eculizumab
CN h5G1.1
CN h5G1.1VHC+h5G1.1VLC
CN Soliris
FS PROTEIN SEQUENCE
MF Unspecified
CI MAN
SR US Adopted Names Council (USAN)
LC STN Files: ADISINSIGHT, BIOSIS, CA, CAPLUS, CBNB, CHEMCATS, EMBASE, IMSDRUGNEWS, IMSPATENTS, IMSPRODUCT, IMSRESEARCH, IPA, MRCK*, PHAR, PROUSDDR, TOXCENTER, USAN, USPATFULL
(*File contains numerically searchable property data)

*** STRUCTURE DIAGRAM IS NOT AVAILABLE ***
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59 REFERENCES IN FILE CA (1907 TO DATE)
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Opposition against EP 1720571
(Application No.: 05 71 2607.0)
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Our Ref.: M2367 OPP(EP)

München, December 30, 2013
WA/ARO/FLG

This is in response to the Official Communication of April 19, 2013 issued by the European Patent Office in the above-referenced opposition proceedings.

It is requested that the patent EP-B1 1720571 is maintained on the basis of the claims as granted. As will be evident from our written submissions herein below, our request that the patent be maintained on the basis of the granted claims is fully justified. Since furthermore the Opponent, Pfizer Inc., formally and irrevocably has withdrawn its opposition on October 7, 2013, it is requested that the opposition proceedings are promptly terminated.

In case the Opposition Division would be inclined to proceed differently, we request oral proceedings in accordance with Article 116 EPC. In that case we request that we be allowed to submit one or more additional claim request(s).

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1. INADMISSIBLE BROADENING (Articles 100(c) and 123(2) EPC)

1.1. The then Opponent alleges that the subject-matter of independent claims 1 and 5 and the claims dependent thereon is not directly and unambiguously disclosed by the application a filed thereby contravening Article 123(2) EPC.

1.2. The second medical use claims 1 and 5 comprise identical features. Claim 1 is drafted as purpose-limited product claim in accordance with the EPC 2000 and claim 5 is drafted as a Swiss-type claim in accordance with the EPC 1973.

The Opponent has divided these claims into the following features:

(1) the use of eculizumab,
(2) the treatment of a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH),
(3) intravenous administration,
(4) administration of 600 mg each week for four weeks,
(5) administration of 900 mg 1 week later,
(6) administration of 900 mg on a biweekly basis,
(7) a breakthrough in serum hemolytic activity when treated under a dosing schedule according to (4) to (6), and
(8) administration of 900 mg every 12 days.

1.2.1. In more detail, the opponent is of the view that each of features (1) and (3) requires the selection of an item from a list of equally categorized items. In other terms, three selections from three lists are allegedly required in order to arrive at features (1) and (3) thereby violating Article 123(2) EPC.

1.2.2. The Opponent furthermore purports that feature (3) has no basis in the application as filed but only the more narrow feature "intravenous infusion by injection" (see page 20, lines 4-8 of the application as filed).

1.2.3. The Opponent considers that the general specification of the applications does not describe any specific dosage regimen. The Opponent concludes that the only basis for features (4) to (8) can be found in the Examples of the application as filed. However, the Opponent contends that features (4) to (8) are an undue

generalisation of the specific contents of the Examples and that in particular the following essential features of the Examples would have been omitted in claims 1 and 5:

(i) the PNH patients are transfusion-dependent and hemolytic

(ii) the meaning of transfusion dependency in the context of the application

(iii) the infusion time of approx. 30 min, and

(iv) the occurrence of the breakthrough between weeks 12 and 64.

1.2.4. With regard to feature (4) the Opponent further alleges that the term "600 mg each week for four weeks" only requires the administration of 600 mg once per week for four weeks, while the Examples would require that the 600 mg are administered at a time interval of exactly 7 days (i.e. weekly intervals) for four weeks.

1.2.5. Finally, the Opponent believes that it would not be clearly supported in the Examples whether the dosages according to features (5) and (6) were intravenously administered. The route of administration would not have been specified in the Examples.

1.3. <u>The new claims have a clear and unambiguous basis in the application as filed.</u>

1.3.1. Granted claim 1 reads:

> "Eculizumab for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein eculizumab is to be administered intravenously to the patient under the following dosing schedule:
> (i) 600 mg each week for four weeks;
> (ii) 900 mg 1 week later;
> (iii) 900 mg on a biweekly basis; and thereafter
> (iv) 900 mg every 12 days,
> wherein said patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii)."

As will be detailed herein below, granted claim 1 and consequently also granted claim 5 has a clear and unambiguous basis in the application as filed, because

(i) all features of these claims can be directly and unambiguously derived from the Examples of the application as filed, and

(ii) no technically essential feature of the examples has been omitted or changed.

In the following we will provide a clear and unambiguous basis for each of features (1) to (8) as classified by the Opponent.

1.3.2. *Feature (1) - use of eculizumab*

Eculizumab is the antibody which is used in the examples of the application as filed; see page 32, line 14 reading

> "The specific antibody used in the study was eculizumab."

Thus - in contrast to the allegations of the opponent - no selection from a list is required in order arrive at the anti-C5 antibody which is eculizumab. Hence, feature (1) is directly and unambiguously supported by the Examples of the application as filed.

1.3.3. *Feature (2) - treatment of a patient afflicted with PNH*

The Examples of the application as filed at page 32 start to read:

> "Eleven patients participated in therapy trials to evaluate the effects of anti-C5 antibody on PNH and symptoms associated therewith. PNH patients were transfusion-dependent and hemolytic. Patients were defined as transfusion dependent with a history of four or more transfusions within twelve months." (emphasis added)

Hence - contrary to the assertions of the opponent - no selection from a list is required in order to define the patient to be treated as patient being afflicted with PNH.

The Opponent further considers that the PNH patient has to be further defined as (i) being hemolytic and (ii) having a history of four or more transfusions within twelve months whereas these features are not reflected by the claim.

(i) hemolytic anemia

At page 2, lines 9 to 15 of the application as filed it is described that PNH is characterized by hemolytic anemia. Hemolytic anemia is one of the characterizing symptoms of the disease PNH.

The Patentee has informed us that every individual to a very slight extent has some PNH cells, but they are present at an extremely low level and of course these individuals are not considered to be PNH patients. In a very small number of individuals these PNH clones of cells begin to expand and when they expand enough, hemolysis becomes apparent. These individuals will be diagnosed as being a PNH patient. Consequently, every individual given a clinical diagnosis of PNH will show hemolysis.

In summary, a PNH patient is inherently hemolytic. The omission of an inherent feature cannot violate Article 123(2) EPC. Compliance of Article 123(2) EPC has to be assessed from the perspective of a skilled person.

(ii) history of blood transfusions

As is evident from the above-citation from page 32 of the application as filed, the PNH patients used in the examples of the application had a history of four or more blood transfusions within twelve months. In order to judge whether this information on the PNH patients has to be considered a technical feature which cannot be omitted in claims 1 and 5 without violating Article 123(2) EPC we refer to the pertinent case law. In T 331/87 the Board developed the following general three criteria for the replacement or removal of a feature from a claim:

> "The replacement or removal of a feature from a claim may not violate Article 123(2) EPC provided the skilled person would directly and unambiguously recognise that
> (1) the feature was not explained as essential in the disclosure,
> (2) it is not, as such, indispensable for the function of the invention in the light of the technical problem it serves to solve, and
> (3) the replacement or removal requires no real modification of other features to compensate for the change (cf. point 6 of the Reasons; following Decision T 260/85, "Coaxial connector/AMP" OJ EPO 1989, 105)." (see headnote of T 331/87)

As regards requirement (1) of the above-described three-point test, we note that nothing in the application as filed conveys the impression that only PNH patients being transfusion-dependent can be treated by eculizumab.

In view of requirement (2) we note that administration of eculizumab prevents hemolytic events by blocking the complement cascade. Since all PNH patients suffer from hemolytic anemia, all PNH patients will benefit from the claimed use of eculizumab. It is of further note that the skilled person immediately understands from the Examples of the application as filed that all PNH patients showing a complement breakthrough should be treated by 900 mg of eculizumab every 12 days. A requirement of a certain transfusion history for the treatment to be successful is not apparent.

With regard to requirement (3) Patentee asserts that all PNH patients and not only transfusion dependent PNH patients may exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii). In more detail, the Patentee has indicated that, to the best of its knowledge, the ability to show breakthrough appears to be independent of the severity of the disease. Instead, breakthrough is related to how rapidly eculizumab is cleared from the circulation. Some patients seem to clear eculizumab at a higher rate than other patients. The severity of the disease - which may be expressed at least in part by the blood transfusion history of a PNH patient - has therefore no technical effect on the other features of granted claims 1 and 5.

It is thus our firm believe that the PNH patient in claims 1 and 5 neither has to be further defined as (i) being hemolytic nor as (ii) having a history of four or more transfusions within twelve months in order to render these claims compliant with Article 123(2) EPC.

1.3.4. *Feature (3) - administered intravenously*

In the Examples on page 32, lines 12-16 it is stated:

> "Over the course of four weeks, each of 11 patients received a weekly 600 mg intravenous infusion of anti-C5 antibody for approximately thirty

> minutes. The specific anti-C5 antibody used in the study was eculizumab. Patients received 900 mg of eculizumab 1 week later then 900 mg on a biweekly basis." (emphasis added)

Hence, in the Examples eculizumab is administered intravenously. Again - contrary to the statements of the opponent - no selection from any list in the specification is required in order to define that eculizumab is administered intravenously.

The allegation of the Opponent that only the first dosage of weekly 600mg for four weeks has been administered intravenously makes no technical sense. As the Opposition Division knows, an application has to be read with a mind willing to understand; see, e.g., decision T 190/99. Also the prior art cited by the opponent itself shows that eculizumab is routinely administered intravenously. For example, it is stated in D4 that

> "Patients received 600mg eculizumab by intravenous infusion over 30 minutes weekly for 4 doses and then 900 mg every other week". (emphasis added)

Essentially corresponding statements can be found in D3 and D6.

Granted claims 1 and 5 refer to intravenous administration and not intravenous infusion. The skilled person knows, though, that an intravenous administration is always by a "push" - which is a one-time, rapid injection of the medication into the bloodstream – or by an "infusion" - which is a slow drip of medication into the vein over a period of time. The technical effect is in both cases that eculizumab is delivered in the blood stream. The only difference is the time frame in which the complete dose of eculizumab is administered. However, the time is not characterized as being essential in the application as filed. The indication of the time is also certainly not indispensable in order to work the invention, and varying the infusion time does not require a modification of the other features of the claim. Hence, in connection with the omittance of the intravenous time the criteria of the "three-point test" developed in T 331/87 are clearly met. Omittance of the infusion time of approx. 30 minutes does not constitute a violation of Article 123(2) EPC.

1.3.5. *Feature (7) - breakthrough in serum hemolytic activity*

The study described in the Examples of the application as filed has been initially carried out for 12 weeks and then has been extended to 64 weeks (see, for example, Table 1 or Figure 3 of the application as filed). The breakthrough in 2 out of 11 PNH patients has been observed between weeks 12 and 64 of the study (see Figure 4 of the application as filed).

It is not essential with respect to the claimed dosage regimen when a PNH patient has a complement breakthrough. The essential step is to increase the dosage from 900mg/14d to 900mg/12d once a complement breakthrough is observed. As it is described in the Examples at page 38, lines 15-17, the complement blockade was permanently restored by shortening the interval of administration - although both PNH patients likely did not show the complement breakthrough at the same time point during the 64-weeks study.

Hence, it is not technically necessary to define in granted clams 1 and 5 at which particular point in time a breakthrough is observed in a PNH patient to be treated.

1.3.6. *Features (4) to (6) and (8)*

The fact that also the dosages according to features (5) and (6) were administered intravenously has been discussed herein above in connection with feature (3).

The Opponent's interpretation of the wording of feature (4) is a mere play on words and should not attract any attention.

Beyond these points the Opponent apparently does not contest that features (4) to (6) and (8) as such can be clearly and unambiguously derived from the Examples of the application as filed. Having said that, the Opponent alleges that these features would only find support in connection with further features of the Examples. These further allegedly omitted features have been discussed herein above in connection with features (1) to (3) and (7).

As is evident from the above discussion in connection with features (1) to (3) and (7) these features do not violate Article 123(2) EPC.

1.3.7. The Opponent does not consider that the features in the dependent claims give raise to any further violation of Article 123(2) EPC. Hence, all of the granted claims comply with Article 123(2) EPC.

2. PRIORITY (Article 87 EPC)

2.1. For essentially the same reasons put forward by the Opponent in section 4 entitled "added subject-matter", the Opponent believes that the subject-matter of the claims cannot be directly and unambiguously derived from the priority application.

The Opponent concludes that the effective date of the granted claims is not the priority date of February 3, 2004 but the international filing date of February 3, 2005.

2.2. As will be shown in the following the relevant contents in the Examples of the application as filed - which form a clear and unambiguous basis for the granted claims - are also comprised in the priority document US 10/771,552; compare page 30, lines 17-18 of the priority document and page 32, lines 16-20 of the application as filed.

In more detail, page 30 of US 10/771,552 reads:

> 5 **EXAMPLES**
>
> Eleven patients participated in therapy trials to evaluate the effects of anti-C5 antibody on PNH and symptoms associated therewith. PNH patients were transfusion-dependent and hemolytic. Patients were defined as transfusion dependent with a history of four or more transfusion within twelve months. The

and

> Over the course of four weeks, each of 11 patients received a weekly 600 mg intravenous infusion of anti-C5 antibody for approximately thirty minutes. The specific anti-C5 antibody used in the study was eculizumab. Patients received 900 mg of eculizumab 1 week later then 900 mg on a bi-weekly basis. The first twelve weeks of the study constituted the pilot study. All patients participated in an extension study conducted to a total of 48 weeks.

These two passages give rise to priority for features (1) to (6).

Page 30 of US 10/771,552 reads

> 0.001). As seen in Figure 4, break-through of complement blockade resulted in hemoglobinuria, dysphagia, and increased LDH and AST. At the next dose, symptoms resolved (Fig 5) and reduction from 900mg every 14 days to 900mg every 12 days resulted in a regain of complement control which was maintained for over 9 months in both patients. This patient shows a 24 hour resolution of

and page 34 of US 10/771,552 reads

> eculizumab treatment. Two patients exhibited a breakthrough in complement activity, but complement blockade was permanently restored by reducing the dosing interval to 12 days (See, Figure 4).

These two passages give rise to priority for features (7) and (8).

Otherwise arguments in favour of clear and unambiguous support in the application as filed as detailed in section 1, *supra* apply *mutatis mutandis* for entitlement of priority.

Hence, the subject-matter of granted claims 1 and 5 has also a clear and unambiguous basis in the priority application. The priority claim is valid.

3. NOVELTY (Articles 100(a) and 54 EPC)

3.1. The Opponent considers that the claims lack novelty in view of D1.

3.2. D1 has been published on February 5, 2004 and hence 2 days after the priority date of the patent. D1 is not prior art because the claims are entitled to the priority date; see section 2, *supra*.

4. INVENTIVE STEP (Article 100(a) and 56 EPC)

4.1. The Opponent alleges that the granted claims lack inventive step in view of D5 considered alone or in combination with any one of D2, D3, D4 or D6. The Opponent also contends that the granted claims are no inventive in view of any one of D2, D3, D4 or D6.

4.2. D5 may be considered as closest prior art in the assessment of inventive step of the granted claims. D5 describes:

> "We previously reported that eculizumab was very effective at acutely controlling intravascular hemolysis in a 3-month Pilot study of transfusion-dependent PNH patients. We now report that all 11 patients from the initial study entered an extension trial of 900mg eculizumab by i.v. infusion over 30 minutes every other week for one year. [...] Two patients experienced transient breakthroughs in complement activity associated with LDH, hemoglobinuria and mild symptoms; these resolves immediately upon next dose of eculizumab and further breakthroughs were prevented by a slight decrease in dosing interval." (emphasis added)

Details on the 3-months pilot study are, for example, described in D3, D4 and D6 (see page 53, left column). Hence, D5 (in combination with any one of D3, D4 and D6) could be seen as describing that 2 out of 11 PNH patients exhibited a complement breakthrough when treated under a dosage regimen similar, but not identical to that recited in the granted claims. D5 furthermore teaches that the complement breakthrough can be permanently prevented by a slight decrease in dosing interval.

4.3. Objective technical problem

Independent granted claims 1 and 5 are distinguished from D5 in that

(i) the dosing interval is the administration of 900 mg eculizumab every 12 days; and

(ii) feature (5) requiring "administration of 900 mg 1 week later" is absent from D5.

Accordingly, the objective technical problem underlying the granted claims in view of D5 may be seen as the determination of the specific dosing interval which prevents further breakthroughs in patients having the prior treatment history as defined in the claims.

The solution is the administration of 900 mg eculizumab every 12 days to patients having the prior treatment history as defined in the claims.

4.4. Non-obviousness

D5 neither teaches nor suggests the time interval of administration which is designated as a "slight decrease in dosing interval". Patentee submits that the skilled person would not have arrived at the exact dose of 900 mg eculizumab every 12 days without the need of inventive skills.

This is particularly evident when considering the prior art at the time of the invention in a comprehensive manner. Pursuant to the pertinent case law, e.g. T 24/81, second headnote, in the examination of inventive step all previously published embodiments must be taken into consideration which offered a suggestion to the skilled practitioner for solving the problem addressed, even where those embodiments were not particularly emphasized.

We enclose an article of Maria Kaplan in Current Opinion on Investigational Drugs 2002, 3(7):1472-4472. This article has been cited as document D10 during examination proceedings of the opposed patent and is introduced into the present opposition proceedings as document D13.

D13 describes at page 4, left column, fourth paragraph a Phase II trail on the treatment of RA patients with eculizumab:

> "Patients were treated with placebo, eculizumab (8 mg/kg iv) once per week for 4 weeks and then once every month (induction/monthly group); eculizumab (8mg/kg iv) one per week for 4 weeks followed by once every 2 weeks (induction/biweekly group); or eculizumab (8 mg/kg iv) once every 2 weeks (biweekly group). The results after 3 months of treatment showed that the induction/monthly group met the primary endpoint of the trial (improvement in American College of Rheumatology response criteria score (ACR) 20 after 3 months of treatment), while the induction/biweekly and biweekly groups did not statistically meet the endpoint."

Importantly, only patients receiving treatment every month, but not the patients receiving treatment every two weeks meet the endpoint of the trial – being an improvement of RA. In other words, an increase of the dose of eculizumab by increasing the dosing intervals did not have a beneficial effect. Hence, D13 shows that shortening of dosing intervals may not result in an effective treatment by eculizumab.

The inventors of the opposed patent have unexpectedly found that the 12-days period is sufficient to successfully treat PNH patients which suffer from breakthrough in serum hemolytic activity when treated under a dosing schedule according to the above-defined features (4) to (6), and at the same time is not too short such that an effective treatment by eculizumab is prevented, as observed in D13. This surprising key finding of the present invention is described at page 35, lines 5-23 of the application as filed:

> "Serum hemolytic activity in nine of the eleven patients was completely blocked throughout the 64 week treatment period with trough levels of eculizumab at equilibrium ranging from approximately 35 µg/ml to 350 µg/ml. During the extension study, 2 patients did not sustain levels of eculizumab necessary to consistently block complement. This breakthrough in serum hemolytic activity occurred in the last 2 days of the 14 day dosing interval, a pattern that was repeated between multiple doses. In one of the patients, as seen in FIG. 4, break-through of complement blockade resulted in hemoglobinuria, dysphagia, and increased LDH and AST, which correlated with the return of serum hemolytic activity. At the next dose, symptoms resolved (FIG. 5) and reduction in the dosing interval from 900 mg every 14 days to 900 mg every 12 days resulted in a regain of complement control which was maintained over the extension study to 64 weeks in both patients. This patient showed a 24 hour resolution of dysphagia and hemoglobinuria. A reduction in the dosing interval from 14 to 12 days was sufficient to maintain levels of eculizumab above 35 µg/ml and

> effectively and consistently blocked serum hemolytic activity for the remainder of the extension study for both patients." (emphasis added)

Hence, the critical point in order to consistently block serum hemolytic activity is maintaining levels of eculizumab above 35 µg/ml. Thus is achieved in accordance with the invention by the reduction of the dosing interval from 900 mg every 14 days to 900 mg every 12 days. Arriving at the 12-days period is consequently not a matter of routine but required due consideration and mindful experimentation. Applicant therefore submits that the granted claims are inventive over D5. The deficiencies of D5 cannot be cured by any one of D2, D3, D4 or D6. No prior art document teaches or suggests a 12-days dosing interval, let alone the superior efficacy in preventing complement breakthrough hemolysis in PNH patients associated with this dosing interval.

5. SUFFICIENCY OF DISCLOSURE (Articles 100(b) and 83 EPC)

5.1. Eculizumab

5.1.1. The Opponent purports that the claims lack sufficiency of disclosure, because the complete sequence of eculizumab (or h5G1.1) would neither be disclosed by the application as filed nor any one of the prior art documents cited by the Opponent.

5.1.2. The Patentee asserts that the sequence for eculizumab was publicly available prior to the priority and filing date. A sequence for eculizumab was submitted to Chemical Abstracts Services (CAS) and entered into their STN database on 14 February 1999; please see the attached copy of the CAS entry No. 219685-560-4.

5.2. The term "breakthrough in serum hemolytic activity"

5.2.1. The Opponent considers that the term "breakthrough in serum hemolytic activity" is not sufficiently disclosed by the application as filed, because it is allegedly not

> "[...] clear whether one, two, three, four of these events [breakthrough of complement blockade] are necessary and/or sufficient to characterize a

breakthrough according to the claims." (see the Opponent's Appeal Brief at section 7.2, end of second paragraph)

The Opponent concludes that the skilled person would not be able to characterize and identify a "breakthrough in serum hemolytic activity".

5.2.2. The argument put forward by the Opponent reads on a lack of clarity rather than on a lack of sufficiency of disclosure. An alleged lack of clarity is not a valid ground for opposition.

Notwithstanding we wish to submit that Figure 4 of the application as filed shows that a complement breakthrough can be identified by blood in the morning urine. Breakthrough in serum hemolytic activity which is the result of the complement activity can also be diagnosed using a blood sample (see page 35, lines 5-14 of the application as filed). As such, and for the mere sake of arguments we submit that the term "breakthrough" is clear for the skilled person and means for its determination are at the skilled person's disposal.

6. CONCLUSION

In view of the above we submit that the claims as granted fulfill the requirements of the EPC. Our request that the patent be maintained on the basis of the granted claims is therefore fully justified.

Dr. Joachim Wachenfeld
European Patent Attorney

Enclosure:
CAS entry
D13

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Date
14-01-2014

Reference	Application No./Patent No.
M2367 EP	05712607.0 - 1412 / 1720571
Applicant/Proprietor	
ALEXION PHARMACEUTICALS, INC.	

Summons to attend oral proceedings pursuant to Rule 115(1) EPC

You are hereby summoned to attend oral proceedings arranged in connection with the above-mentioned European patent.

The matters to be discussed are set out in the communication accompanying this summons (EPO Form 2906).

The oral proceedings, which will be public, will take place before the opposition division

on 15.05.14 at 09.15 hrs in Room 1662 at the EPO,
Grasserstr. 2, PschorrHöfe, D-80335 München

No changes to the date of the oral proceedings can be made, except on serious grounds (see OJ EPO 1/2009, 68). If you do not appear as summoned, the oral proceedings may continue without you (R. 115(2) EPC).

Your attention is drawn to Rule 4 EPC, regarding the language of the oral proceedings, and to the Special edition No. 3 OJ EPO 2007, 128, concerning the filing of authorisations for company employees and lawyers acting as representatives before the EPO.

The final date for making written submissions and/or amendments (R. 116 EPC) is 17.03.14.

You are requested to report in good time beforehand to the porter in the EPO foyer. Room 1601 and 1657 are available as waiting rooms.

Parking is available in the underground car park, accessible only via the entrance "Grasserstrasse 2/6". On presentation of the summons to oral proceedings at the porters' lodge in the main foyer "Bayerstrasse", the parking ticket will be revoked.

1st Examiner:	2nd Examiner:	Chairman:
Chapman R	Weikl M	Cilensek Z

For the Opposition Division

Annexes:
Confirmation of receipt (Form 2936)
Rule 4 EPC (EPC Form 2043)
Communication (EPO Form 2906)

Registered letter with advice of delivery
EPO Form 2310 12.12 [ORAL03=1662] (10/01/14)

ORAL4

to EPO postal service: 10.01.14

CC20871

1 Facts and Submissions

Reference is made to the following documents cited by the Opponent; the numbering will be adhered to for the rest of the proceedings.

D1 Hillmen et al., (2004) The New England Journal of Medecine, 350:552-9

D2 Jane Salodof MacNeil (2002) Medscape Medical News

D3 Hillmen et al., (2003) British Journal of Hematology 121 (Suppl. 1) 87-95, Abstract 257

D4 Hillmen et al., (2002) Blood. 100(11), Abstract 154

D5 Hillmen et al., (2003) Journal of The American Society of Hematology 102(11) Abstract 1858

D6 Rother et al., (2003) Molecular Immunology. 40, p 197, Abstract 54

D7 Rosse et al., (2004) American Society of Hematology, p 48-62

D8 Biotechnology News (2004) 24(30), p 10

D9 US 6,355,245

D10 Thomas et al., (1996) Molecular Immunology, Vol. 33, No. 17/18, p 1389-1401

D11 Drugs of the Future (2004) 29(7): 673-676

D12 Hillmen et al., British Journal of Haematology, 125 (Suppl. 1), 1-72, Abstract 5

1.1 European patent EP1720571 (hereinafter designated the patent) entitled "Method of treating hemolytic disease", was published in the official bulletin on 13.06.2012 and a corrigendum was issued on 24.10.2012. The Proprietor (hereafter designated 'P') is Alexion Pharmaceuticals, Inc., Cheshire, US.

Said patent is based on European application 05712607.0, filed on 03.02.2005 and claims the priority from US 10/771,552 filed on 03.02.2004. The International publication number is WO2005/074607.

1.2 Notice of opposition against the above-mentioned patent was received from the following party (hereinafter named the Opponent or 'O').

1. Pfizer Inc., 235 East 42nd Street, New York 10017, US (filed online with letter dated 12.03.2013, received on 12.03.2013).

1.3 The O requested the revocation of the patent as granted in its entirety on the basis of Articles 100(a)(b)(c) because the subject-matter granted was not novel, inventive, or disclosed in a manner sufficiently clear and complete to be

carried out by a skilled person (Articles 52(1),54,56 and 83 EPC), and comprised subject-matter that extended beyond the application as originally filed (Article 123(2) EPC). The O filed D1-D12 therewith.

1.4 In their letter dated 07.10.2013, the O withdrew their request of opposition against the above-mentioned patent.

1.5 With their letter dated 09.10.2013, the P requested that their patent be maintained as granted and that the opposition procedure be closed.

1.6 The P requested oral proceedings in case their respective request would not be granted.

2 Preliminary, non-binding opinion of the opposition division (OD)

The admissibility of the opposition has not been contested by the P. The opposition is thus considered admissible (Article 99(1) and Rule 76 EPC).

The O submitted documents and arguments that would appear to be *prima facie* prejudicial to the maintenance of the patent as granted. Since the O has withdrawn his opposition, the OD feels it pertinent to continue opposition *proceedings upon its own volition* (GL D, VII-5.2,5.3; Article 114(1) EPC).

Added subject-matter (Articles 100(c) and 123(2) EPC)

2.1 The O objected to claims 1 and 5 of the patent on grounds that they are not directly and unambiguously disclosed in the application, rather an intermediate generalisation of a specific example (p.32-33) combined with general teachings of the application.

2.2 The O considered the claims to contain the following features:

1. the use of eculizumab
2. the treatment of a patient affected with PNH
3. I.V. administration
4. administration of 600mg each week for 4 weeks
5. administration of 900mg a week later
6. administration of 900mg bi-weekly thereafter
7. a breakthrough in serum hemolytic activity when the patient is treated under a dosing schedule according to points 4 - 6
8. administration of 900mg every 12 days thereafter

2.3 Features 1 and 2 would appear to have a general basis on p.12, li.1-3 and p. 18, li.12-20, respectively. The Examples on p.33 also contain these features.

2.4 Feature 3 would appear to be broader than the basis provided on p.20, li.4-8, which relates merely to 'intravenous infusion by injection'. Feature 4 would also encompass both injection into a vein using a needle and the use of a catheter. The OD finds that since p.20, li.4-8 neither specifically states the use of a needle or a catheter, 'intravenous infusion by injection' could be considered to encompass both I.V. infusion via needle or catheter, i.e. intravenous administration; however, 'I.V. administration' does not necessarily imply administration of an infusion. 'Infusion' implies the use of a catheter, but this is not stated in the application. The OD is thus of the opinion that **Feature 3 has no basis** in the application as filed as it also encompasses administration of pharmaceutical formulations other than infusions.

2.5 Features 4 - 7 relate to a dosage schedule. The general teaching of the application on p.19-20 states that the dose will vary according to the compound used, the manner of administration, the symptoms, the size and the age of the patient. The only basis for the dosage regimen in the claim comes from the specific example on p.32-33, which describes steps 1 - 7 applied to a patient group. Two of the patients in that group exhibited breakthrough in serum hemolytic activity which was controlled by reducing the dosage interval to 12 days (p.35, li.8-17). The O is of the opinion that the combination of Features 1 - 8 cannot be derived from either the general part of the description or the examples.

2.6 The OD agrees that the only basis for claims 1 and 5 is in the specific examples.

2.7 However, the O argues that 'essential features' of the example are missing from the claim. The OD notes that clarity is not a grounds of opposition. There is no indication in the application to suggest that the specific patient sub-group to be treated must be transfusion-dependent and hemolytic (p.32, li.7-9), that an infusion time of 'approximately 30 mins' (p.30, li.12-14), or that the time point of breakthrough occurrence (between weeks 12-64) are essential to the application claimed. The OD finds that it is clear from the examples that any patient receiving the claimed dosage regimen for PNH and experiencing breakthrough could be treated by shortening the interval between doses, as claimed. The OD does not see that there is an intermediate generalisation of the examples in this respect.

2.8 The O further argues that there is no basis for the route of administration used following the initial four week dosing schedule, i.e. Feature 4, since the route thereafter is not specified. The OD finds that it would be obvious that further antibody treatments would be administered by the same route, however, the O is correct in that this is not clearly stated in the application. This point will require further discussion at oral proceedings.

Lack of novelty (Articles 100(a) and 54 EPC)

2.9 The O raised an objection on grounds of lack of novelty regarding claims 1 and 5 with regard to D1.

2.10 D1 published on 05.02.2004 discloses all the features of claims 1-8 (see Methods and section 'Biochemical indicators of hemolysis' on p.552-556 and Fig.3A) but is published after the date of priority of the patent.

2.11 The patent claims priority from US10/771552, filed on 03.02.2004, and appears to disclose identical subject-matter to the application filed on 03.02.2005 on which the patent is based.

2.12 The O considered the priority document not to disclose the subject-matter of the present claims for the same reasons they consider the patent's claims not to have a basis in the application as filed (Article 123(2) EPC).

2.13 Since doubt has been raised as to whether a basis for claims 1 and 5 exists in the application, the same doubts result for the disclosure of the priority document. Therefore, the subject-matter of D1 is considered relevant for the assessment of the novelty and inventive step of the claims.

2.14 The OD is of the provisional opinion that claims 1-8 of the contested patent lack novelty over D1.

Lack of inventive step

2.15 The O objected to the patent on grounds of a lack of inventive step, taking D2, D3,D4 or D6 as the closest prior art to the subject matter of claims 1 and 5.

2.16 Documents D3,D4 and D6 all disclose the following features:

1. the use of eculizumab
2. the treatment of a patient affected with PNH
3. I.V. administration
4. administration of 600mg each week for 4 weeks

5. administration of 900mg a week later
6. administration of 900mg bi-weekly thereafter

2.17 Document D2 does not disclose the Feature 3 and thus will not be considered further.

2.18 The difference between either D3,D4 or D6 and the present claims is considered to be the dosage.

2.19 The technical effect associated with this difference is the alleviation of symptoms associated with a breakthrough in hemolytic activity.

2.20 The problem to be solved can therefore be considered to be the provision of an improved dosage regimen for patients suffering a breakthrough in hemolytic activity.

2.21 The solution provided in the granted claims would appear obvious, since according to D5, transient breakthroughs in complement activity associated with increased LDL, hemoglobinuria and mild symptoms in an extension trial of patients receiving the dosage regimen described in D3,D4 or D6 were known to be preventable by a slight decrease in the dosing interval.

2.22 The skilled person with a knowledge of any of D3,D4 or D6, and observing a breakthrough in hemolytic activity in patients would therefore find it obvious to slightly reduce the dosing interval until no further hemoglobinuria event is observed (as shown in Fig.5 of the patent).

2.23 The establishment of optimal dosage regimens is considered to be routine according to the case law of the Technical Board of Appeal T1979/09, wherin under point 7.3.1 it is stated *'It is well known that in designing dosage regimen the two major parameters that can be adjusted are the dose itself, i.e. the quantity of drug administered, and the dosing frequency, i.e. the time interval between doses.'*

2.24 and further under point 7.3.2, *'In the present case the amount of active ingredient remains the same as the amount used in D5 and there is no evidence on file of any unexpected technical effect caused by administering the red vine leave extract once daily. The board considers that it is within the competence of the skilled person to find out the optimal dosage regime. Consequently, the replacement of a dosage regime by another dosage regime for the same purpose is considered to be a matter of routine experimentation and cannot be seen as involving an inventive step.'*

2.25 It is therefore the provisional opinion of the OD that at least the subject-matter of claims 1 and 5 does not involve an inventive step, in light of either D5 alone, or in combination with any one of D3,D4 or D6.

2.26 Dependent claims 2-4 and 6-8 do not appear to contain any additional features which, in combination with any other claim to which they refer, would meet the requirements of the EPC with respect to inventive step.

Insufficiency of disclosure (Articles 100(b) and 83 EPC)

2.27 The O raised the objection that the trade name 'eculizumab' did not sufficiently disclose such an antibody, since, at the time of filing, such an antibody was not completely described.

2.28 The application makes reference to D9 and D10, which do not make reference to deposits or sequences that disclose the humanised anti-C5 antibody to which it allegedly refers.

2.29 The OD is of the opinion, that in the absence of evidence that eculizumab *could be identified as a specific antibody, either by deposit or* ***full*** *sequence of* its light and heavy chains, the antibody and thus the subject-matter of claims 1-8 cannot be considered to be sufficiently disclosed.

2.30 The O made further objections to the clarity of the term 'breakthrough in serum hemolytic activity'. The OD will not pursue this objection since a lack of *clarity is not a ground for opposition.*

Conclusion

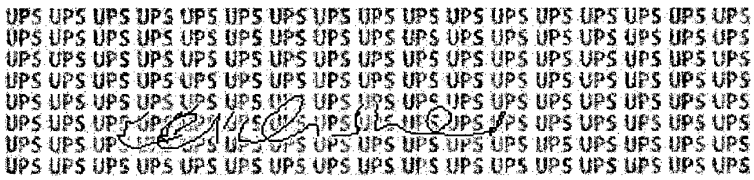
3 At present the OD is of the preliminary opinion that the patent contains added subject-matter (Articles 100(c) and 123(2) EPC), does not involve an inventive step (Articles 100(a) and 56 EPC), and lacks sufficient disclosure (Article 100 (b) and 83 EPC).

In view of the request of the P, oral proceedings are hereby appointed.

Tracking Information

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Reference	Application No./Patent No.
M2367 EPOPPCEP)	05712607.0 - 1412 / 1720571
Applicant/Proprietor	
ALEXION PHARMACEUTICALS, INC.	

EPA/EPO/OEB Formblatt/Form/Formulaire : 2310

Empfangsbescheinigung über den Zugang des vorstehend bezeichneten Schriftstücks
Acknowledgement of receipt of the document specified above
Récépissé du document spécifié ci-dessus

Unter Bezugnahme auf die Mitteilung im ABl EPA 7/2010, 377 wird gebeten, die Empfangsbescheinigung mit Empfangsdatum und Unterschrift zu versehen und **umgehend** an das EPA zurückzusenden:

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Conformément au communiqué paru au JO OEB 7/2010, 377, vous êtes prié d'indiquer sur le récépissé la date de réception du document, de signer le récépissé et de le renvoyer **sans délai** à l' OEB:

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Reference	Application No./Patent No.
M2367 EP	05712607.0 - 1412 / 1720571

Applicant/Proprietor
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BRIEF COMMUNICATION

Subject:
- ☒ Your letter of 30.12.13
- ☐ Our telephone conversation of
- ☒ Communication of 14.01.14
- ☐

Enclosure(s):
- ☐ Letter from the proprietor of the patent of
- ☐ Letter from the opponent of
- ☐ Copy (copies)

Communication:

☐ A letter from the opponent /proprietor was received on ..... . The documents specified as patent documents in this letter are available via the European Patent Register at **www.epo.org/register.** Should you wish to receive these documents in paper format, they will be supplied to you free of charge if specifically requested on receipt of this communication (OJ EPO 2009, 434).

☒ Please find an additional statement by the Opposition Division in the annex.

For the Opposition Division

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☐ **Registered letter**

EPO Form 2911O 01.12 (18/02/14)

CC20871

The following opinion incorporates the P's response to the O's objections, that were not available to the OD at the time of issuance of the summons to oral proceedings.

The following non-binding preliminary opinion serves to augment those objections raised in the summons to Oral Proceedings.

The P further cited the following document:

D13 KAPLAN M: "ECULIZUMAB", CURRENT OPINION IN INVESTIGATIONAL DRUGS, PHARMAPRESS, US, vol. 3, no. 7, 1 July 2002 (2002-07-01), pages 1017-1023, XP009045356, ISSN: 1472-4472

Added subject-matter (Articles 100(c) and 123(2) EPC)

1.1 The O objected to claims 1 and 5 of the patent on grounds that they are not directly and unambiguously disclosed in the application, rather an intermediate generalisation of a specific example (p.32-33) combined with general teachings of the application.

1.2 The O considered the claims to contain the following features:

1. the use of eculizumab
2. the treatment of a patient affected with PNH
3. I.V. administration
4. administration of 600mg each week for 4 weeks
5. administration of 900mg a week later
6. administration of 900mg bi-weekly thereafter
7. a breakthrough in serum hemolytic activity when the patient is treated under a dosing schedule according to points 4 - 6
8. administration of 900mg every 12 days thereafter

1.3 Features 1 and 2 would appear to have a general basis on p.12, li.1-3 and p. 18, li.12-20, respectively. The Examples on p.33 also contain these features.

1.4 Feature 3 would appear to be broader than the basis provided on p.20, li.4-8, which relates merely to 'intravenous infusion by injection'. Feature 4 would also encompass both injection into a vein using a needle and the use of a catheter.

1.5 The P refers to the Examples on p.32, li.12-16 for a basis. This passage refers to 'intravenous infusion of anti-C5 antibody'. The P also states that 'intravenous administration', referred to in granted claims 1 and 5 is not the same as 'intravenous administration', the former referring to a one-time rapid injection of the medication into the blood stream and the latter to a slow drip of medication into the vein. The P argues, however, that the technical effect is the same, the only difference being the time frame in which the dose is administered.

1.6 The OD finds that since p.20, li.4-8 neither specifically states the use of a needle or a catheter, 'intravenous infusion by injection' could be considered to encompass both I.V. infusion via needle or catheter, i.e. intravenous administration; however, 'I.V. administration' does not necessarily imply administration of an infusion. 'Infusion' implies the use of a catheter, but this is not stated in the application.

1.7 The OD is thus of the opinion that **Feature 3 has no basis** in the application as filed, and as confirmed by the P, as it also encompasses administration of pharmaceutical formulations other than infusions.

1.8 Features 4 - 7 relate to a dosage schedule. The general teaching of the application on p.19-20 states that the dose will vary according to the compound used, the manner of administration, the symptoms, the size and the age of the patient. The only basis for the dosage regimen in the claim comes from the specific example on p.32-33, which describes steps 1 - 7 applied to a patient group. Two of the patients in that group exhibited breakthrough in serum hemolytic activity which was controlled by reducing the dosage interval to 12 days (p.35, li.8-17).

1.9 The O is of the opinion that the combination of Features 1 - 8 cannot be derived from either the general part of the description or the examples.

1.10 The P argues that the basis for claims 1 and 5 is in the specific examples.

1.11 The OD agrees with the view of the P, since all essential features of the examples would appear to be in said claims.

1.12 However, the O argues that 'essential features' of the example are missing from the claim. The OD notes that clarity is not a grounds of opposition. There is no indication in the application to suggest that the specific patient sub-group to be treated must be transfusion-dependent and hemolytic (p.32, li.7-9), that an infusion time of 'approximately 30 mins' (p.30, li.12-14), or that the time point of breakthrough occurrence (between weeks 12-64) are essential to the application claimed. The OD finds that it is clear from the examples that any

patient receiving the claimed dosage regimen for PNH and experiencing breakthrough could be treated by shortening the interval between doses, as claimed.

1.13 The OD does not see that there is an intermediate generalisation of the examples in this respect.

1.14 The O further argues that there is no basis for the route of administration used following the initial four week dosing schedule, i.e. Feature 4, since the route thereafter is not specified. The OD finds that it would be obvious that further antibody treatments would be administered by the same route, however, the O is correct in that this is not clearly stated in the application. This point will require further discussion at oral proceedings.

Lack of novelty (Articles 100(a) and 54 EPC)

1.15 The O raised an objection on grounds of lack of novelty regarding claims 1 and 5 with regard to D1.

1.16 D1 published on 05.02.2004 discloses all the features of claims 1-8 (see Methods and section 'Biochemical indicators of hemolysis' on p.552-556 and Fig.3A) but is published after the date of priority of the patent.

1.17 The patent claims priority from US10/771552, filed on 03.02.2004, and appears to disclose identical subject-matter to the application filed on 03.02.2005 on which the patent is based.

1.18 The O considered the priority document not to disclose the subject-matter of the present claims for the same reasons they consider the patent's claims not to have a basis in the application as filed (Article 123(2) EPC).

1.19 The P argues that the claims have a basis in both the application and priority document, and therefore the subject-matter is new.

1.20 Since the OD doubts that a basis for claims 1 and 5 exists in the application regarding feature 3, the same doubts result for the disclosure of the priority document. Therefore, the subject-matter of D1 is considered relevant for the assessment of the novelty and inventive step of the claims.

1.21 The OD is of the provisional opinion that claims 1-8 of the contested patent lack novelty over D1.

1.22 The P should understand that if a basis can be found, or amendments made to remove the objections regarding added subject-matter, the objections on grounds of lack of novelty will also be overcome.

Lack of inventive step

1.23 The O objected to the patent on grounds of a lack of inventive step, taking D2, D3,D4 or D6 as the closest prior art to the subject matter of claims 1 and 5.

1.24 Documents D3,D4 and D6 all disclose the following features:

1. the use of eculizumab
2. the treatment of a patient affected with PNH
3. I.V. administration
4. administration of 600mg each week for 4 weeks
5. administration of 900mg a week later
6. administration of 900mg bi-weekly thereafter

1.25 Document D2 does not disclose the Feature 3 and thus will not be considered further.

1.26 The difference between either D3,D4 or D6 and the present claims is considered to be the dosage.

1.27 The technical effect associated with this difference is the alleviation of symptoms associated with a breakthrough in hemolytic activity.

1.28 The problem to be solved can therefore be considered to be the provision of an improved dosage regimen for patients suffering a breakthrough in hemolytic activity.

1.29 The solution provided in the granted claims would appear obvious, since according to D5, transient breakthroughs in complement activity associated with increased LDL, hemoglobinuria and mild symptoms in an extension trial of patients receiving the dosage regimen described in D3,D4 or D6 were known to be preventable by a slight decrease in the dosing interval.

1.30 The skilled person with a knowledge of any of D3,D4 or D6, and observing a breakthrough in hemolytic activity in patients would therefore find it obvious to slightly reduce the dosing interval until no further hemoglobinuria event is observed (as shown in Fig.5 of the patent).

1.31 The P provides a similar problem-and-solution approach, but starting from D5 and combining with any of D3,D4 or D6. A similar problem is posed by the P, albeit that the problem they suggest is formulated as a 'determining a dosing interval' rather than the provision of a medical use.

1.32 The P states that the skilled person starting from D5 would not decrease the time interval to 12 days as claimed, since said D5 refers only to 'a slight decrease in dosing interval'. The P refers to D13 (cited in examination as D10), p.1020, col.1, §4, wherein a Phase II trial is described. The P notes that the only regimen to reach the primary endpoint was a weekly/monthly regimen, whereas weekly/bi-weekly and biweekly regimens did not. Although, the OD notes that the same passage states that both weekly/monthly and weekly/biweekly groups met the secondary endpoint after 3 months of therapy. The P concludes that the skilled person would conclude from D13 that shortening of dosing intervals may not result in an effective treatment by eculizumab, and that in accordance with T24/81 this prior art should be considered alongside all other published embodiments.

1.33 The OD considers that D13 is not directed to the same technical problem posed. The claim is clearly directed to patients suffering breakthrough who are already receiving a standard regimen. The standard regimen has been described in D2,D3,D4 and D6, and D5 clearly discloses how cases in which breakthrough is experienced can be resolved. D5, as with all these prior art documents was published after D13 and represent the actual stand of knowledge at the time of priority filing. The OD therefore considers that given the nature of D13 in reporting preliminary research results, and the subsequent publications D2-D6, the skilled person would not consider D13 to teach increasing dosing intervals as opposed to decreasing them (as in D5).

1.34 The OD further notes that the establishment of optimal dosage regimens was considered to be routine according to the case law of the Technical Board of Appeal T1979/09, wherein under point 7.3.1 it is stated *'It is well known that in designing dosage regimen the two major parameters that can be adjusted are the dose itself, i.e. the quantity of drug administered, and the dosing frequency, i.e. the time interval between doses.'*

1.35 and further under point 7.3.2, *'In the present case the amount of active ingredient remains the same as the amount used in D5 and there is no evidence on file of any unexpected technical effect caused by administering the red vine leave extract once daily. The board considers that it is within the competence of the skilled person to find out the optimal dosage regime. Consequently, the replacement of a dosage regime by another dosage regime for the same purpose is considered to be a matter of routine experimentation and cannot be seen as involving an inventive step.'*

1.36 It is therefore the provisional opinion of the OD that at least the subject-matter of claims 1 and 5 does not involve an inventive step, in light of either D5 alone, or in combination with any one of D3,D4 or D6.

1.37 Dependent claims 2-4 and 6-8 do not appear to contain any additional features which, in combination with any other claim to which they refer, would meet the requirements of the EPC with respect to inventive step.

Insufficiency of disclosure (Articles 100(b) and 83 EPC)

1.38 The O raised the objection that the trade name 'eculizumab' did not sufficiently disclose such an antibody, since, at the time of filing, such an antibody was not completely described.

1.39 The application makes reference to D9 and D10, which do not make reference to deposits or sequences that disclose the humanised anti-C5 antibody to which it allegedly refers.

1.40 The P states that eculizumab was publicly available prior to priority filing: its sequence was available through CAS on the STN database since 14.02.1999 (CAS 219685-560-4).

1.41 The OD is of the opinion, that the antibody and thus the subject-matter of claims 1-8 can be considered to be sufficiently disclosed as the antibody was available to the skilled person at the time of priority filing.

1.42 The O made further objections to the clarity of the term 'breakthrough in serum hemolytic activity'. The OD will not pursue this objection since a lack of clarity is not a ground for opposition.

Conclusion

2 At present the OD is of the preliminary opinion that the patent contains added subject-matter (Articles 100(c) and 123(2) EPC), and does not involve an inventive step (Articles 100(a) and 56 EPC).

The scheduled date for Oral Proceedings is thus maintained.

R. Chapman for the O.D.

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17. März 2014

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80298 M U N I C H

EP 05 71 2607.0 / 1720571
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Our Ref.: M2367 OPP(EP)

München, March 17, 2014
WA/ARO/FLG

This is in response to the Summons to attend oral proceedings issued on January 14, 2014 and the Brief Communication issued on February 18, 2014 by the European Patent Office in the above-referenced opposition proceedings.

We enclose a new main request and a new auxiliary request which replace the claims as granted of the opposed patent EP-B1 1720571. As it will be evident from the following, both new main requests fulfill the requirements of the EPC. In case patentability of the main request cannot be accepted, consideration of the auxiliary request is requested.

1. AMENDMENTS TO THE CLAIMS (Rule 137(4) EPC)

1.1. Main Request

1.1.1. New claim 1 is based on claim 1 as granted. New claim 5 is based on claim 5 as granted. The term "intravenously" has been replaced by the phrase "by intravenous infusion". This amendment is supported at page 32, line 13 of the

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application as filed. Further support can be found at page 20, line 6 of the application as filed.

1.1.2. New claims 2-4 and 6-8 correspond to claims 2-4 and 6-8 as granted.

1.2. Auxiliary Request

1.2.1. New claim 1 is based on claim 1 of the main request. New claim 5 is based on claim 5 as granted. The phrase "wherein cell lysis is reduced to below 20% of normal serum complement activity when treated under a dosing schedule according to items (i) to (iv)" has been added. This amendment is supported by page 38, lines 11-17 and Figure 8 of the application as filed.

1.2.2. New claims 2-4 and 6-8 correspond to claims 2-4 and 6-8 of the main request.

2. INADMISSIBLE BROADENING (Articles 100(c) and 123(2) EPC)

2.1. The feature "Intravenous administration" of claim 1

2.1.1. The Opposition Division is of the preliminary view that the feature "intravenous administration" of claim 1 (i.e. feature 3 as characterized by the Opposition Division) extends beyond the contents of the application as filed, because it may also encompass administration by means other than infusions.

2.1.2. New claims 1 and 5 of the main request have been amended as described above. In more detail, the term "intravenously" has been replaced by the phrase "by intravenous infusion". The same amendment is present in claims 1 and 5 of the auxiliary request. The phrase "by intravenous infusion" has a clear and unambiguous basis at page 32, line 13 and page 20, line 6 of the application as filed.

We therefore submit that new claims 1 and 5 of the main request as well as auxiliary request comply with Article 123(2) EPC.

2.2. Mode of administration of the dosage schedule

2.2.1. The Opponent has argued that there is no basis in the application as filed that the dosages following the administration of 600mg eculizumab each week for 4 weeks are administered by the same route. The Opposition Division states that it is obvious that also the remainder of the administration steps are administered by the same route. However, since the Opponent's observation that no

administration route is expressly mentioned in connection with the further administration steps is correct, the Opposition Division wishes to further discuss this point at the oral proceedings.

2.2.2. In preparation of the discussion of this issue at the oral proceedings, we wish to provide the following comments in writing. We fully concur with the Opposition Division's statement that it is obvious that also the further administration steps are administered by the same route. The disputed passage at page 32 of the application reads:

> "Over the course of four weeks, each of 11 patients received a weekly 600 mg intravenous infusion of anti-C5 antibody for approximately thirty minutes. The specific anti-C5 antibody used in the study was eculizumab. Patients received 900 mg of eculizumab 1 week later then 900 mg on a biweekly basis. The first twelve weeks of the study constituted the pilot study." (emphasis added)

Nothing in this passage conveys the impression that the mode of administration via intravenous infusion is changed after the initial dosing step of 600mg eculizumab per week for four weeks. The allegation of the Opponent that only the first dosage of weekly 600mg for four weeks has been administered by intravenous infusion makes no sense. In fact, if the mode of administration were to be changed, a corresponding statement would be expected in the corresponding passages. This is, however, not the case. The only mode referred to in the examples is intravenous infusion.

In fact, the attitude of the skilled person is to read the contents of a patent specification with a mind willing to understand the technical sense, not a mind desirous of misunderstanding (T 190/99; confirmed *inter alia* in T 1204/06 or T 1771/06). Although this case law on how a skilled person reads the contents of an application has been established in connection with Article 84 EPC, the Boards have established essentially the same standard under Article 123(2) EPC. In this regard reference is made to T 609/95, reasons for the decision 2.3:

> "The board takes the position that where a drafting defect or inconsistency in an application would be evident to a reader skilled in the art, the person to whom the application is addressed, it is reasonable to suppose that he would, in the light of the content of the application, attempt to formulate a notional amendment which would enable him to make sense of what he reads, and to the extent that the amendment might be said to leap to the mind of the reader, although perhaps only after close study of the document, it can be regarded as implicit in the application and would not contravene Article 123(2) EPC, if effected in practice." (emphasis added)

Such standard applies all the more to the present case, given that there is no drafting defect or inconsistency. The principle that the skilled person tries to sensibly interpret the contents of the application also with regard to claim amendments has been confirmed in T 887/97, reasons for the decision 2.2. Hence, also when it comes to the assessment of the contents of the application under Article 123(2) EPC, the skilled person aims at making sense of what he reads in an application.

It is furthermore emphasized that the application is addressed to the skilled person and hence the contents of the application as filed have to be assessed from the perspective of the skilled person. The skilled person unequivocally reads the contents of the above-quoted passage of the application as filed in the light of the available knowledge at the priority date of the opposed patent.

In view of the above-recited case law, the skilled person cannot understand the above-quoted passage from the application other than that eculizumab is administered by intravenous infusion throughout the entire study, especially in view of the fact that no different mode of administration is mentioned in the Examples.

2.3. In summary we submit that the new claims of the main and auxiliary request have a clear and unambiguous basis in the application as originally filed.

3. NOVELTY (Articles 100(a) and 54 EPC)

3.1. The Opposition Division is of the preliminary view that feature 3 of granted claim 1 and arguably also the mode of administration of the dosage schedule have no proper basis in the priority application. For this reason, the Opposition Division is of the view that the claims are no entitled to priority and consequently D1 becomes prior art.

The Opposition Division states that D1 anticipates claims 1 to 8.

3.2. The feature "Intravenous administration" of claim 1

The new claims have been amended as described above. In more detail, the term "intravenously" has been replaced by the phrase "by intravenous infusion". The skilled person can clearly and unambiguously derive the mode of "intravenous infusion" from page 30, line 14 and page 19, line 17 of the priority-establishing application.

3.3. Mode of administration of the dosage schedule

The passage at page 32, lines 12-15 of the application as filed corresponds to the contents of the priority-establishing application at page 30, lines 13-19.

In G 1/03, G 2/03 and G 2/10 the Enlarged Board held that the disclosure as the basis for the right to priority under Article 87(1) EPC, and as the basis for amendments in an application under Article 123(2) EPC have to be interpreted in the same way. Hence, for the same reasons set forth in section 2.2.2, *supra* it is immediately evident to the skilled person – in the sense that nothing else can be meant - that eculizumab is administered by intravenous infusion in the entire study as described in the priority-establishing application.

3.4. With regard to the auxiliary request it is to be added that the phrase "wherein cell lysis is reduced to below 20% of normal serum complement activity when treated under a dosing schedule according to items (i) to (iv)" can be clearly and unambiguously derived from page 34, lines 13-19 and Figure 8 of the priority document.

3.5. We therefore submit that the skilled person can immediately derive the subject-matter of the new main and auxiliary request from the priority-establishing application. The time rank of the new claims is consequently February 3, 2004; i.e. before D1 became available to the public. D1 is not prior art for the new set of claims.

4. INVENTIVE STEP (Articles 100(a) and 56 EPC)

4.1. The Opposition Division is of the preliminary view that at least claims 1 and 5 lack an inventive step in view of D5 optionally combined with D3, D4 or D6.

The Opposition Division defines the technical problem underlying the claimed subject-matter as the provision of an improved dosage regimen of eculizumab.

The solution of this technical problem - i.e. the administration of 900mg eculizumab every 12 days instead of every 14 days – is allegedly obvious, because D5 teaches that transient complement breakthrough can be prevented by a slight decrease in dosing interval.

The Opposition Division thus takes the position that a skilled person starting from D5 would have reduced the dosing interval until no complement

breakthrough is observed. By referring to decision T 1979/09, reasons for the decision sections 7.3.1 and 7.3.2, the Opposition Division emphasizes that finding the optimal dosage interval cannot justify the presence of an inventive step.

4.2. Our arguments indicating the presence of an inventive step submitted on December 31, 2013 are fully maintained and still applicable to the enclosed new main and auxiliary requests.

In order to underline our position that the skilled person starting from D5, optionally combined with D3, D4 or D6, would not have arrived at the exact dose of 900 mg eculizumab every 12 days without the need of inventive skills, the following further arguments are provided.

4.2.1. D5 teaches:

> "Two patients experienced transient breakthroughs in complement activity associated with increased LDH, hemoglobinuria and mild symptoms; these resolved immediately upon next dose of eculizumab and further breakthroughs were prevented by a slight decrease in the dosing interval." (emphasis added)

For a proper assessment of inventive step, we would like to elaborate on the following issues: What would the skilled person have observed upon slightly decreasing the interval, in particular from 14 days to 13 days? Would he/she have been encouraged to further decrease the interval?

4.2.2. The attention of the Opposition Division is respectfully drawn to Figure 4 of the application as filed. Figure 4 shows hemolysis tests on urine samples of the studied PNH patients. In more detail, in the urine samples the levels of lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) were measured. LDH and AST are often used in the art as a marker of hemolysis, as LDH and AST are abundant in red blood cells. Upon red blood cell lysis LDH and AST are released from the cell to the extracellular compartments of the body. Consequently, LDH and AST can be detected in a urine sample if red blood cell lysis occurs. In the absence of red blood cell lysis LDH and AST cannot be detected in a urine sample. It follows that the more LDH or AST is found in the urine sample, the more hemolysis occurs.

The pharmacokinetics (PK) and pharmacodynamics (PD) of eculizumab were also measured. PK is a measure of the concentration of eculizumab in the serum of a patient over time and PD is a measure of the level of complement activity in the patient's serum. The method used for the determination of PD levels is described at page 37, line 10 to page 38, line 10 of the application as filed. High PD levels indicate high levels of complement activity in the patient's serum, which in turn correlates with high levels of hemolysis.

The urine samples tested in Figure 4 have been obtained from PNH patients who received a dose of 900mg eculizumab every 14 days. The day on which 900mg eculizumab was administered is characterized as day "0" in Figure 4. Urine samples were obtained at day "0" and each day between day "9" and day "13" of the thirteen days between the mentioned fortnightly administrations of 900mg eculizumab.

At day 0, i.e. before the administration of eculizumab, LDH, AST and PD levels were consistently high, thereby indicating the occurrence of hemolysis in the tested PNH patients. LDH, AST and PD levels again were measured on days 9, 12 and 13 after administration of eculizumab. The measured LDH, AST, and PD consistently show that no hemolysis was found until day 12. However, on day 13, LDH, AST and PD levels surprisingly increased dramatically, thereby indicating an abrupt relapse of hemolytic activity in the PNH patients. In particular, the PD levels as shown in Figure 4 demonstrate the dramatic increase of serum hemolytic activity from day 12 to day 13:

	Day 12	Day 13	Day 14 = day 0[1]
PD	2	56	56

(excerpt of contents of Figure 4 of the application as filed)

Hence, the data in Figure 4 of the application as filed evidence that the administration of 900mg eculizumab every 12 days – as claimed – is the optimum dosage regimen for the administration of eculizumab. Assuming that 900mg eculizumab would be administered every 13 days, complement

[1] We note that day 14 is designated day 0 in Figure 14, given that the next dose is administered.

breakthrough may be still observed in PNH patients. If 900mg eculizumab were administered every 11 days, this would be an unnecessarily short interval which is of no further benefit for the PNH patients. Hence, Figure 4 evidences that the optimal time window for the administration of 900mg eculizumab is very narrow. In fact, the claimed 12 days dosing interval is ideal, while administration every 11 or 13 days cannot be viewed as ideal dosing intervals.

Importantly, the data in Figure 4 evidence that a decrease from 14 to 13 days is not encouraging. The breakthrough patients would remain in the realm of high LDH and unfavorable PD.

4.3. Since the Opposition Division relies on T 1979/09, reasons for the decision 7.3.1 and 7.3.2, for concluding that finding an optimal dosage interval may justify the presence of an inventive step, we will demonstrate in the following that the case decided in T 1979/09 has to be held distinct from the present situation.

4.3.1. *Dosage range vs. exact dosage*

It can be taken from reason for the decision 7.1 that the claims at stake in T 1979/09 did not refer to an exact dosage but to a broad dosage range of 300mg to 800mg of the extract in 1 to 3 capsules of tablets taken once daily. Thus, irrespective of whether 300mg of the extract or 800mg of the extract are administered as daily treatment, success is ensured.

On the other hand, claim 1 of the enclosed new main and auxiliary request refers to a distinct dose of 900mg eculizumab at a distinct time interval of every 12 days. As it has been detailed in section 4.2, *supra*, the precisely and narrowly defined dosing scheme according to step (iv) of claim 1 of the enclosed new main and auxiliary request – being the administration of 900mg eculizumab every 12 days – ensures successful treatment of PNH breakthrough patients. As such the facts of the case underlying in T 1979/09 on the one hand and the present case on the other hand are fundamentally different. It was by no means obvious from D5 optionally combined with D3, D4 or D6 for the skilled person to arrive at the mentioned narrow and specific dosing schedule.

4.3.2. *Closest prior art in T 1979/09 and in the present case*

We consider it also important to note that the prior art in T 1979/09 was considerably closer to the claimed subject-matter as in the present case. As it is evident from reason for the decision 7.3, the prior art taught to administer a total daily dose of 360mg to 540mg (which is within the claimed range of 300mg to 800mg) of the extract in 2 to 3 capsules (which is within the claimed range of 1 to 3 capsules) twice a day. It follows that in T 1979/09 the claimed total daily dose of the extract comprised that of the prior art. The only difference is that the dose is administered once a day instead of twice.

In the present case D5 may be seen as the closest prior art document. D5 merely mentions a "slight decrease in dosing interval" but fails to suggest any specific interval. Hence, the gap between D5 and the claimed subject-matter is indeed wider than the gap between the closest prior art and the claimed subject-matter in the case at issue in T 1979/09. The skilled person was not able to close the gap in the present situation without the need of inventive skills. D5 conveys the impression that a slight decrease of the dosing interval of 14 days would prevent complement breakthrough. However, as set forth in section 4.2 of this petition a decrease from 14 days to 13 days – which certainly is much closer to a "slight decrease" than from 14 to 12 days - fails to prevent further complement breakthroughs. As detailed herein above, administering 900mg eculizumab at an interval of every 12 days ensures optimal treatment.

This was by no means foreseeable for the skilled person on the basis of D5 optionally combined with D3, D4 or D6. None of these documents guides the skilled person to the interval of 12 days.

4.3.3. *Presence of an unexpected technical effect*

Section 7.3.2 of the Reasons for the decision of T 1979/09 reads:

> "In the present case the amount of active ingredient remains the same as the amount used in D5 [i.e. the closest prior art document in T 1979/09] and there is no evidence on file of any unexpected technical effect caused

> by administering the red vine leave extract once daily." (information in brackets and emphasis added)

Hence, in T 1979/09 no technical advantage associated with the distinguishing feature from the closest prior art could be demonstrated.

Moreover, section 7.3.3 of the Reasons for the decision of T 1979/09 teaches:

> "It has never been shown that taking a dose of 300-800 mg once daily has the same effect as taking the same dose twice daily."

As such, it even has not been shown in the case at issue in T 1979/09 that administering the extract once a day is as beneficial as twice a day.

On the other hand, in the present application it has been demonstrated that unexpected technical effects are associated with administering 900mg eculizumab every 12 days. The interval of 12 days is the optimum while shorter as well as longer intervals are associated with the discussed drawbacks. It is our firm belief that the advantageous technical effects being associated with an interval of 12 days fully justify the acknowledgment of an inventive step.

4.4. For the sake of completeness we note with regard to the auxiliary request that the feature of "reducing cell lysis to below 20% of normal serum complement activity" is a feature indicating the effective inhibition of haemolytic activity; see page 38, lines 11-13 and Figure 8. The effective inhibition of haemolytic activity is a key advantage being associated with the subject-matter claimed in the main and auxiliary requests. This advantage is expressly incorporated in the main claim of the auxiliary request.

4.5. In summary, we submit that the enclosed main and auxiliary requests meet the requirements of Article 56 EPC.

5. SUMMARY AND REQUESTS

In view of the above, we submit that the set of claims constituting the main request meets the requirements of the EPC. In case the patentability of the

main request cannot be acknowledged consideration of the auxiliary request is requested.

We further request that in view of our submissions, oral proceedings are cancelled and the proceedings be continued in writing. In case the Opposition Division comes to a different conclusion, we request that we be allowed to submit one or more additional claim request(s) on the occasion of the oral proceedings.

Dr. Joachim Wachenfeld
European Patent Attorney

Enclosure:
New main and auxiliary requests

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Patentanwälte Rechtsanwälte

Via Telefax – 13 pages
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To the immediate attention of
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EP 05 71 2607.0 / 1720571
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Our Ref.: M2367 OPP(EP)

München, March 18, 2014
WA/ARO/FLG

This is further to the response to the Summons to attend oral proceedings submitted in the above-referenced opposition proceedings on March 17, 2014.

Enclosed are copies of the main request and auxiliary request discussed in the above-mentioned response.

Dr. Roger Absehe
European Patent Attorney

Enclosure:
Main and auxiliary requests (track mode and clean copy)

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EP 05 71 2607.0
Alexion Pharmaceuticals, Inc.
Our Ref.: M2367 EP

MAIN REQUEST

1. Eculizumab for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein eculizumab is to be administered by intravenously infusion to the patient under the following dosing schedule:
   (i) 600 mg each week for four weeks;
   (ii) 900 mg 1 week later;
   (iii) 900 mg on a biweekly basis; and thereafter
   (iv) 900 mg every 12 days,
   wherein said patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii).

2. Eculizumab for use according to claim 1, wherein eculizumab is to be administered in combination with one or more compounds that increase hematopoiesis.

3. Eculizumab for use according to claim 2, wherein said one or more compounds that increase hematopoiesis are selected from the group consisting of: steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin, antithymocyte globulin, and antilymphocyte globulin.

4. Eculizumab for use according to any one of claims 1 to 3, wherein the patient is characterized by one or more characteristics selected from the group consisting of:
   (a) the proportion of type III red blood cells of the patient's total red blood cell count is greater than 10%;
   (b) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 25%;

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(c) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 50%;

(d) the patient's platelet count is greater than 40,000 per microliter;

(e) the patient's platelet count is greater than 75,000 per microliter;

(f) the patient's platelet count is greater than 150,000 per microliter;

(g) the patient's reticulocyte count is greater than 80 x 10^9 per liter;

(h) the patient's reticulocyte count is greater than 120 x 10^9 per liter;

(i) the patient's reticulocyte count is greater than 150 x 10^9 per liter;

(j) the patient has a greater than 40% type III granulocyte clone; and

(k) the patient has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

5. Use of eculizumab in the preparation of a medicament for treating a patient with paroxysmal nocturnal hemoglobinuria (PNH), wherein eculizumab is to be administered by intravenously infusion to the patient under the following dosing schedule:

(i) 600 mg each week for four weeks;

(ii) 900 mg 1 week later;

(iii) 900 mg on a biweekly basis; and thereafter

(iv) 900 mg every 12 days,

wherein said patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii).

6. The use according to claim 5, wherein eculizumab is to be administered in combination with one or more compounds that increase hematopoiesis.

7 The use according to claim 6, wherein said one or more compounds that increase hematopoiesis are selected from the group consisting of: steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin, antithymocyte globulin, and antilymphocyte globulin.

8. The use according to any one of claims 5 to 7, wherein the patient is characterized by one or more characteristics selected from the group consisting of:

(l) the proportion of type III red blood cells of the patient's total red blood cell count is greater than 10%;

(m) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 25%;

(n) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 50%;

(o) the patient's platelet count is greater than 40,000 per microliter;

(p) the patient's platelet count is greater than 75,000 per microliter;

(q) the patient's platelet count is greater than 150,000 per microliter;

(r) the patient's reticulocyte count is greater than 80×10^9 per liter;

(s) the patient's reticulocyte count is greater than 120×10^9 per liter;

(t) the patient's reticulocyte count is greater than 150×10^9 per liter;

(u) the patient has a greater than 40% type III granulocyte clone; and

(v) the patient has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

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Alexion Pharmaceuticals, Inc.
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MAIN REQUEST

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   (i) 600 mg each week for four weeks;
   (ii) 900 mg 1 week later;
   (iii) 900 mg on a biweekly basis; and thereafter
   (iv) 900 mg every 12 days,
   wherein said patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii).

2. Eculizumab for use according to claim 1, wherein eculizumab is to be administered in combination with one or more compounds that increase hematopoiesis.

3. Eculizumab for use according to claim 2, wherein said one or more compounds that increase hematopoiesis are selected from the group consisting of: steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin, antithymocyte globulin, and antilymphocyte globulin.

4. Eculizumab for use according to any one of claims 1 to 3, wherein the patient is characterized by one or more characteristics selected from the group consisting of:
   (a) the proportion of type III red blood cells of the patient's total red blood cell count is greater than 10%;
   (b) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 25%;

(c) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 50%;

(d) the patient's platelet count is greater than 40,000 per microliter;

(e) the patient's platelet count is greater than 75,000 per microliter;

(f) the patient's platelet count is greater than 150,000 per microliter;

(g) the patient's reticulocyte count is greater than 80 x 10^9 per liter;

(h) the patient's reticulocyte count is greater than 120 x 10^9 per liter;

(i) the patient's reticulocyte count is greater than 150 x 10^9 per liter;

(j) the patient has a greater than 40% type III granulocyte clone; and

(k) the patient has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

5. Use of eculizumab in the preparation of a medicament for treating a patient with paroxysmal nocturnal hemoglobinuria (PNH), wherein eculizumab is to be administered by intravenous infusion to the patient under the following dosing schedule:

(i) 600 mg each week for four weeks;

(ii) 900 mg 1 week later;

(iii) 900 mg on a biweekly basis; and thereafter

(iv) 900 mg every 12 days,

wherein said patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii).

6. The use according to claim 5, wherein eculizumab is to be administered in combination with one or more compounds that increase hematopoiesis.

7 The use according to claim 6, wherein said one or more compounds that increase hematopoiesis are selected from the group consisting of: steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin, antithymocyte globulin, and antilymphocyte globulin.

8. The use according to any one of claims 5 to 7, wherein the patient is characterized by one or more characteristics selected from the group consisting of:

(l) the proportion of type III red blood cells of the patient's total red blood cell count is greater than 10%;

(m) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 25%;

(n) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 50%;

(o) the patient's platelet count is greater than 40,000 per microliter;

(p) the patient's platelet count is greater than 75,000 per microliter;

(q) the patient's platelet count is greater than 150,000 per microliter;

(r) the patient's reticulocyte count is greater than 80 x 10^9 per liter;

(s) the patient's reticulocyte count is greater than 120 x 10^9 per liter;

(t) the patient's reticulocyte count is greater than 150 x 10^9 per liter;

(u) the patient has a greater than 40% type III granulocyte clone; and

(v) the patient has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

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Alexion Pharmaceuticals, Inc.
Our Ref.: M2367 OPP(EP)

AUXILIARY REQUEST

1. Eculizumab for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein eculizumab is to be administered by intravenous infusion to the patient under the following dosing schedule:

   (i) 600 mg each week for four weeks;

   (ii) 900 mg 1 week later;

   (iii) 900 mg on a biweekly basis; and thereafter

   (iv) 900 mg every 12 days,

   wherein said patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii), and wherein cell lysis is reduced to below 20% of normal serum complement activity when treated under a dosing schedule according to items (i) to (iv).

2. Eculizumab for use according to claim 1, wherein eculizumab is to be administered in combination with one or more compounds that increase hematopoiesis.

3. Eculizumab for use according to claim 2, wherein said one or more compounds that increase hematopoiesis are selected from the group consisting of: steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin, antithymocyte globulin, and antilymphocyte globulin.

4. Eculizumab for use according to any one of claims 1 to 3, wherein the patient is characterized by one or more characteristics selected from the group consisting of:

   (a) the proportion of type III red blood cells of the patient's total red blood cell count is greater than 10%;

   (b) the proportion of type III red blood cells of the patient's total red blood

cell content is greater than 25%;

(c) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 50%;

(d) the patient's platelet count is greater than 40,000 per microliter;

(e) the patient's platelet count is greater than 75,000 per microliter;

(f) the patient's platelet count is greater than 150,000 per microliter;

(g) the patient's reticulocyte count is greater than 80 x 10^9 per liter;

(h) the patient's reticulocyte count is greater than 120 x 10^9 per liter;

(i) the patient's reticulocyte count is greater than 150 x 10^9 per liter;

(j) the patient has a greater than 40% type III granulocyte clone; and

(k) the patient has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

5. Use of eculizumab in the preparation of a medicament for treating a patient with paroxysmal nocturnal hemoglobinuria (PNH), wherein eculizumab is to be administered by intravenous infusion to the patient under the following dosing schedule:

(i) 600 mg each week for four weeks;

(ii) 900 mg 1 week later;

(iii) 900 mg on a biweekly basis; and thereafter

(iv) 900 mg every 12 days,

wherein said patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii), and wherein cell lysis is reduced to below 20% of normal serum complement activity when treated under a dosing schedule according to items (i) to (iv).

6. The use according to claim 5, wherein eculizumab is to be administered in combination with one or more compounds that increase hematopoiesis.

7 The use according to claim 6, wherein said one or more compounds that increase hematopoiesis are selected from the group consisting of: steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin,

antithymocyte globulin, and antilymphocyte globulin.

8. The use according to any one of claims 5 to 7, wherein the patient is characterized by one or more characteristics selected from the group consisting of:

(l) the proportion of type III red blood cells of the patient's total red blood cell count is greater than 10%;

(m) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 25%;

(n) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 50%;

(o) the patient's platelet count is greater than 40,000 per microliter;

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(q) the patient's platelet count is greater than 150,000 per microliter;

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Our Ref.: M2367 OPP(EP)

AUXILIARY REQUEST

1. Eculizumab for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein eculizumab is to be administered by intravenous infusion to the patient under the following dosing schedule:
   (i) 600 mg each week for four weeks;
   (ii) 900 mg 1 week later;
   (iii) 900 mg on a biweekly basis; and thereafter
   (iv) 900 mg every 12 days,
   wherein said patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii), and
   wherein cell lysis is reduced to below 20% of normal serum complement activity when treated under a dosing schedule according to items (i) to (iv).

2. Eculizumab for use according to claim 1, wherein eculizumab is to be administered in combination with one or more compounds that increase hematopoiesis.

3. Eculizumab for use according to claim 2, wherein said one or more compounds that increase hematopoiesis are selected from the group consisting of: steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin, antithymocyte globulin, and antilymphocyte globulin.

4. Eculizumab for use according to any one of claims 1 to 3, wherein the patient is characterized by one or more characteristics selected from the group consisting of:
   (a) the proportion of type III red blood cells of the patient's total red blood cell count is greater than 10%;
   (b) the proportion of type III red blood cells of the patient's total red blood

cell content is greater than 25%;

(c) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 50%;

(d) the patient's platelet count is greater than 40,000 per microliter;

(e) the patient's platelet count is greater than 75,000 per microliter;

(f) the patient's platelet count is greater than 150,000 per microliter;

(g) the patient's reticulocyte count is greater than 80 x 10^9 per liter;

(h) the patient's reticulocyte count is greater than 120 x 10^9 per liter;

(i) the patient's reticulocyte count is greater than 150 x 10^9 per liter;

(j) the patient has a greater than 40% type III granulocyte clone; and

(k) the patient has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

5. Use of eculizumab in the preparation of a medicament for treating a patient with paroxysmal nocturnal hemoglobinuria (PNH), wherein eculizumab is to be administered by intravenous infusion to the patient under the following dosing schedule:

(i) 600 mg each week for four weeks;

(ii) 900 mg 1 week later;

(iii) 900 mg on a biweekly basis; and thereafter

(iv) 900 mg every 12 days,

wherein said patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii), and

wherein cell lysis is reduced to below 20% of normal serum complement activity when treated under a dosing schedule according to items (i) to (iv).

6. The use according to claim 5, wherein eculizumab is to be administered in combination with one or more compounds that increase hematopoiesis.

7 The use according to claim 6, wherein said one or more compounds that increase hematopoiesis are selected from the group consisting of: steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin,

antithymocyte globulin, and antilymphocyte globulin.

8. The use according to any one of claims 5 to 7, wherein the patient is characterized by one or more characteristics selected from the group consisting of:

(l) the proportion of type III red blood cells of the patient's total red blood cell count is greater than 10%;

(m) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 25%;

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(t) the patient's reticulocyte count is greater than 150 x 10^9 per liter;

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(v) the patient has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

VOSSIUS & PARTNER

Patentanwälte Rechtsanwälte

Via Telefax – 13 pages
Fax- No. 089-2399-4465 and confirmation copy

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

European Patent Office

EPO - Munich
82
18. März 2014

To the immediate attention of
Dr. Chapman

80298 M U N I C H

EP 05 71 2607.0 / 1720571
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Our Ref.: M2367 OPP(EP)

München, March 18, 2014
WA/ARO/FLG

Confirmation Copy

This is further to the response to the Summons to attend oral proceedings submitted in the above-referenced opposition proceedings on March 17, 2014.

Enclosed are copies of the main request and auxiliary request discussed in the above-mentioned response.

Dr. Roger Absehe
European Patent Attorney

Enclosure:
Main and auxiliary requests (track mode and clean copy)

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EP 05 71 2607.0
Alexion Pharmaceuticals, Inc.
Our Ref.: M2367 OPP(EP)

AUXILIARY REQUEST

1. Eculizumab for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein eculizumab is to be administered by intravenous infusion to the patient under the following dosing schedule:
   (i) 600 mg each week for four weeks;
   (ii) 900 mg 1 week later;
   (iii) 900 mg on a biweekly basis; and thereafter
   (iv) 900 mg every 12 days,
   wherein said patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii), and
   wherein cell lysis is reduced to below 20% of normal serum complement activity when treated under a dosing schedule according to items (i) to (iv).

2. Eculizumab for use according to claim 1, wherein eculizumab is to be administered in combination with one or more compounds that increase hematopoiesis.

3. Eculizumab for use according to claim 2, wherein said one or more compounds that increase hematopoiesis are selected from the group consisting of: steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin, antithymocyte globulin, and antilymphocyte globulin.

4. Eculizumab for use according to any one of claims 1 to 3, wherein the patient is characterized by one or more characteristics selected from the group consisting of:
   (a) the proportion of type III red blood cells of the patient's total red blood cell count is greater than 10%;
   (b) the proportion of type III red blood cells of the patient's total red blood

cell content is greater than 25%;

(c) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 50%;

(d) the patient's platelet count is greater than 40,000 per microliter;

(e) the patient's platelet count is greater than 75,000 per microliter;

(f) the patient's platelet count is greater than 150,000 per microliter;

(g) the patient's reticulocyte count is greater than 80 x 10^9 per liter;

(h) the patient's reticulocyte count is greater than 120 x 10^9 per liter;

(i) the patient's reticulocyte count is greater than 150 x 10^9 per liter;

(j) the patient has a greater than 40% type III granulocyte clone; and

(k) the patient has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

5. Use of eculizumab in the preparation of a medicament for treating a patient with paroxysmal nocturnal hemoglobinuria (PNH), wherein eculizumab is to be administered by intravenous infusion to the patient under the following dosing schedule:

(i) 600 mg each week for four weeks;

(ii) 900 mg 1 week later;

(iii) 900 mg on a biweekly basis; and thereafter

(iv) 900 mg every 12 days,

wherein said patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii), and wherein cell lysis is reduced to below 20% of normal serum complement activity when treated under a dosing schedule according to items (i) to (iv).

6. The use according to claim 5, wherein eculizumab is to be administered in combination with one or more compounds that increase hematopoiesis.

7 The use according to claim 6, wherein said one or more compounds that increase hematopoiesis are selected from the group consisting of: steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin,

antithymocyte globulin, and antilymphocyte globulin.

8. The use according to any one of claims 5 to 7, wherein the patient is characterized by one or more characteristics selected from the group consisting of:

(l) the proportion of type III red blood cells of the patient's total red blood cell count is greater than 10%;

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(r) the patient's reticulocyte count is greater than 80 x 10^9 per liter;

(s) the patient's reticulocyte count is greater than 120 x 10^9 per liter;

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(u) the patient has a greater than 40% type III granulocyte clone; and

(v) the patient has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

EP 05 71 2607.0
Alexion Pharmaceuticals, Inc.
Our Ref.: M2367 EP

MAIN REQUEST

1. Eculizumab for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein eculizumab is to be administered by intravenously infusion to the patient under the following dosing schedule:
   (i) 600 mg each week for four weeks;
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   (iii) 900 mg on a biweekly basis; and thereafter
   (iv) 900 mg every 12 days,
   wherein said patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii).

2. Eculizumab for use according to claim 1, wherein eculizumab is to be administered in combination with one or more compounds that increase hematopoiesis.

3. Eculizumab for use according to claim 2, wherein said one or more compounds that increase hematopoiesis are selected from the group consisting of: steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin, antithymocyte globulin, and antilymphocyte globulin.

4. Eculizumab for use according to any one of claims 1 to 3, wherein the patient is characterized by one or more characteristics selected from the group consisting of:
   (a) the proportion of type III red blood cells of the patient's total red blood cell count is greater than 10%;
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5. Use of eculizumab in the preparation of a medicament for treating a patient with paroxysmal nocturnal hemoglobinuria (PNH), wherein eculizumab is to be administered by intravenously infusion to the patient under the following dosing schedule:

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(iv) 900 mg every 12 days,

wherein said patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii).

6. The use according to claim 5, wherein eculizumab is to be administered in combination with one or more compounds that increase hematopoiesis.

7 The use according to claim 6, wherein said one or more compounds that increase hematopoiesis are selected from the group consisting of: steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin, antithymocyte globulin, and antilymphocyte globulin.

8. The use according to any one of claims 5 to 7, wherein the patient is characterized by one or more characteristics selected from the group consisting of:

(l) the proportion of type III red blood cells of the patient's total red blood cell count is greater than 10%;

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   (iv) 900 mg every 12 days,
   wherein said patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii).

2. Eculizumab for use according to claim 1, wherein eculizumab is to be administered in combination with one or more compounds that increase hematopoiesis.

3. Eculizumab for use according to claim 2, wherein said one or more compounds that increase hematopoiesis are selected from the group consisting of: steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin, antithymocyte globulin, and antilymphocyte globulin.

4. Eculizumab for use according to any one of claims 1 to 3, wherein the patient is characterized by one or more characteristics selected from the group consisting of:
   (a) the proportion of type III red blood cells of the patient's total red blood cell count is greater than 10%;
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5. Use of eculizumab in the preparation of a medicament for treating a patient with paroxysmal nocturnal hemoglobinuria (PNH), wherein eculizumab is to be administered by intravenous infusion to the patient under the following dosing schedule:

(i) 600 mg each week for four weeks;

(ii) 900 mg 1 week later;

(iii) 900 mg on a biweekly basis; and thereafter

(iv) 900 mg every 12 days,

wherein said patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii).

6. The use according to claim 5, wherein eculizumab is to be administered in combination with one or more compounds that increase hematopoiesis.

7 The use according to claim 6, wherein said one or more compounds that increase hematopoiesis are selected from the group consisting of: steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin, antithymocyte globulin, and antilymphocyte globulin.

8. The use according to any one of claims 5 to 7, wherein the patient is characterized by one or more characteristics selected from the group consisting of:

(l) the proportion of type III red blood cells of the patient's total red blood cell count is greater than 10%;

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EP 05 71 2607.0
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   wherein said patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii), and
   wherein cell lysis is reduced to below 20% of normal serum complement activity when treated under a dosing schedule according to items (i) to (iv).

2. Eculizumab for use according to claim 1, wherein eculizumab is to be administered in combination with one or more compounds that increase hematopoiesis.

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4. Eculizumab for use according to any one of claims 1 to 3, wherein the patient is characterized by one or more characteristics selected from the group consisting of:
   (a) the proportion of type III red blood cells of the patient's total red blood cell count is greater than 10%;
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5. Use of eculizumab in the preparation of a medicament for treating a patient with paroxysmal nocturnal hemoglobinuria (PNH), wherein eculizumab is to be administered by intravenous infusion to the patient under the following dosing schedule:

(i) 600 mg each week for four weeks;

(ii) 900 mg 1 week later;

(iii) 900 mg on a biweekly basis; and thereafter

(iv) 900 mg every 12 days,

wherein said patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii), and

wherein cell lysis is reduced to below 20% of normal serum complement activity when treated under a dosing schedule according to items (i) to (iv).

6. The use according to claim 5, wherein eculizumab is to be administered in combination with one or more compounds that increase hematopoiesis.

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(u) the patient has a greater than 40% type III granulocyte clone; and

(v) the patient has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

European patent application No. 05712607.0

II. To the Formalities Officer

1. ☒ The date / time fixed for oral proceedings is maintained.

   ☐ (if necessary) The reasons are indicated on enclosed EPO Form 2906/2906O, which is to be dispatched to the party with EPO Form 2008A/2310A.

2. ☐ At the instigation of the division the oral proceedings cannot take place on the arranged date for the reasons indicated on enclosed EPO Form 2906, which is to be dispatched to the party with EPO Form 2008A/2310A.

   Date _____ Director (see Internal Instructions IE-III, 1.2) _____

3. ☐ The summons to attend oral proceedings on ..... should be cancelled.

   3.1 ☐ The proceedings will be continued in writing.

   3.2 ☐ A new date will be set later.

   3.3 ☐ A new date/time is set as follows:

   date ..... at ..... hrs.
   Parties' written submissions and amendments in preparation for the oral proceedings, if any, should be made not later than

   ..... month(s) before the date of the oral proceedings.

4. ☐ The request for the oral proceedings to be held as a videoconference

   4.1 ☐ is allowed.

   4.2 ☐ is rejected for the reasons indicated on enclosed EPO Form 2906, which is to be dispatched to the party with EPO Form 2008A.

5. ☐ The application is deemed to be withdrawn. The oral proceedings are cancelled.

25.03.14
Date

Chairman ✓ Second member Primary Examiner Legal member

III. Action taken by the formalities officer

☒ EPO Form 2008A/2310A has been dispatched, if applicable (see point II above) together with EPO Form 2906/2906O.

☐ A new summons has been dispatched.

☐ .....

25.03.14
Date

C. Cavestri
Formalities Officer

EPO Form 2088 04.10

CC20871

Europäisches Patentamt
European Patent Office
Office européen des brevets

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Questions about this communication ?
Contact Customer Services at www.epo.org/contact

Formalities officer: Mr Consalvo Cavestri

Please specify the name of the formalities officer when contacting Customer Services by contact form or phone.

FAXED

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Date: 30-04-2014

Reference	Application No./Patent No.
M2367 EP	05712607.0 - 1412 / 1720571
Applicant/Proprietor: ALEXION PHARMACEUTICALS, INC.	

BRIEF COMMUNICATION

Oral Proceedings on 15.05.14

Subject: ☒ Your letter of 18.03.14

Communication:
- ☐ The summons to attend oral proceedings on 15.05.14 has been cancelled.
- ☐ The procedure will be continued in writing.
- ☒ The date fixed for oral proceedings is maintained.
- ☐ A new date will be set later.
- ☐

Please take note.

For the Opposition Division

Europäisches Patentamt • European Patent Office • Office européen des brevets

Registered letter
EPO Form 2008A 12.07 (30/04/14)

CC20871

Europäisches Patentamt
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Application No.: 05 712 607.0

Patent No.: EP-B-1 720 571

Minutes of the oral proceedings before the OPPOSITION DIVISION

The proceedings were public.

Proceedings opened on 15.05.2014 at 09:15 hours

Present as members of the opposition division:

Chairman:	Cilensek, Zoran
1st member:	Chapman, Rob
2nd member:	Weikl, Martina
Minute writer:	Weikl, Martina

Present as or for the party or parties:

- For the Proprietor(s): ALEXION PHARMACEUTICALS, INC.

  Wachenfeld, Joachim (European Patent Attorney at Vossius & Partner)
  Abseher, Roger (European Patent Attorney at Vossius & Partner)
  Grasser, Florian (European Patent Attorney at Vossius & Partner)
  accompanied by
  Spalding, Todd N. (Vice President and Head of Intellectual Property at Alexion Pharmaceuticals, Inc.)
  Banks, Damon (Patent Agent at Alexion Pharmaceuticals, Inc

- For the Opponent 1: Pfizer Inc.

The identity of the person/s (as well as, if applicable, that of the witness or witnesses) and, where necessary, the authorisation to represent/authority to act were checked.

Essentials of the discussion and possible relevant statements of the parties:

1 The Chairman (CH) formally opened the Oral Proceedings (OP) for European patent EP1720571 with the title 'Method of Treating Hemolytic Disease' and informed the patentee about the presence of a paralegal assisting the proceedings in writing the minutes but not being part of the Opposition Division (OD) (see OJ 2014, A32).

2 Since the Opponent (O) had withdrawn the opposition with the letter dated 07. October 2013 and the proceedings were continued upon the OD's own volition, only the patentee (P) was represented at the OP.

3 The CH summarized that the opposition had been filed by the opponent Pfizer, Inc. on 12. March 2013 and that grounds of opposition under Articles 100(a), (b) and (c) had been mentioned in the notice of opposition. The CH explained further that the continuation of the opposition proceedings after withdrawal of opposition and upon the volition of the OD was based on the arguments of the opponent.

4 The CH confirmed with the representative (RP) of the P that there was a Main Request (MR) and an Auxiliary Request (AR1) on file, both filed with the letter dated 18. March 2014. The P furthermore requested to submit new documents into the OP before pleading their case. According to the P, these documents were destined to support the P's argumentation with respect to inventive step of the claimed subject-matter. The CH stated the OD would decide upon admission of said documents later in the proceedings, i.e. when the discussion would be turned to Article 56 EPC.

5 The CH then opened the discussion on Article 123(2) EPC and invited the P to indicate the basis under Article 123(2) EPC for the amendments which were present in the current MR (as opposed to the granted claims).

5.1 The P explained that the only amendment had been performed to the claimed route of administration in independent claims 1 and 5 of the MR. Basis for the new definition 'by intravenous infusion' could be found on page 32, line 13 of the description as filed, which related to a dosage regimen in the context of the claimed medical use. The P explained further that the new definition was narrower than the granted definition, hence no objections under Article 123(3) could arise. The P further stated that the amendments were a response to the objection of the O (Rule 80 EPC) and that there was no clarity issue (Article 84 EPC).

5.2 The P enquired whether further explanation concerning the valid combination of the newly introduced feature with the remaining features of the claim was necessary (compare point 1.14 of the summons dated 18. February 2014). The OD confirmed that it would be obvious that further antibody treatments would be administered by the same route and that no further discussion had to be opened on that subject.

5.3 Following the invitation of the CH, the P then submitted documents D14 (Annex 1) and D15 (Annex 2). D14 was a declaration of the inventor L. Bell. It was submitted to support the fact that dosing of the antibody had a specific effect on the health of the patient, and in particular that step (ii) of claims 1 and 5 was important for managing breakthrough patients (see point 4 of D14). D15 was a leaflet distributed with the marketed drug Solaris® (=eculizimab) which under points 2.1 and 5.6 summarized the same dosage regimen as claimed in the independent claims of the current MR. Hence, these documents should be admitted into the proceedings in order to further illustrate the relevance of the currently claimed dosage regimen.

6 After a break, the CH informed the P that the OD was of the opinion that the MR met the requirements of Articles 123(2), 123(3) and 84 EPC as well as of Rule 80 EPC. In addition, and following the line of reasoning as outlined in point 1.22 of the summons dated 18. February 2014, the CH informed the P that the OD was of the opinion that the MR did fulfil the requirements of Article 54 EPC.

7 The CH furthermore stated that D14 and D15 were allowed into the proceedings and opened the discussion on Article 56 EPC. It was agreed that D5 was the closest prior art (CPA).

7.1 The P opened his argumentation by summarizing the contribution to the art of the contested patent. The claimed subject-matter addressed the problem/risk of complement breakthrough in a group of patients undergoing eculizimab therapy for the treatment of Paroxysmal Nocturnal Hemoglobinuria (PNH). The claimed subject-matter was limited to a second-medical use including the dosage regimen required to efficiently overcome these problems. The P furthermore asked the OD to bear in mind that the skilled person in the field was a cautious, conservative person not willing to enter unpredictable areas.

7.2 With that in mind, the P identified two differences between the independent claims of the MR and D5 (which also dealt with the above described problem of breakthrough), the first one being step iv) having a dosing schedule feature of 900 mg every 12 days for patients exhibiting breakthrough whereas D5 did not recite an actual time interval but only disclosed slight decrease in the

dosing interval. The P argued that the interval reduction from 14 to 12 days was a substantial decrease of 14% and could not be referred to as a "slight" decrease.

The second difference was identified by the P as being the dosage regimen prior to the maintenance/extension phase, i.e. the definition of steps corresponding to steps (i) and (ii) of the current independent claims. The P emphasized that D5 itself was silent with respect to the dosage regimen prior to the extension study. According to the P, the subject-matter of steps (i) and (ii) (referred to as 'induction phase by the P) could also not be derived from a combination with other prior art documents which *had* disclosed details of the 'induction phase', such as D3, D4 and D6.

More particularly the P referred to D3 (abstract 257, line 5) and D4 (Abstract 154, line 10) and stated that both documents did not include step ii) but rather related to '900mg every other week'. Upon enquiry by the CH, the P declared that this had to be interpreted such that the first 900mg dose of antibody was given 2 weeks after the last administration of a 600mg dose, and not after one week as required by step (ii) of the claimed schedule.

7.3 The P conceded that D6 described an induction phase identical to steps (i) to (iii) of the claimed dosage regimen. He argued however that this disclosure was outweighed by the two contrasting disclosures of D3 and D4. Furthermore, the skilled person being cautious and conservative (see above) would be likely to choose the combination of the teaching of D5 with the induction phases as disclosed in D3 and D4. Hence, the skilled person would not end up with the dosage regimen as currently claimed. However, as was emphasized in D14, the presence of step (ii) was crucial for the subsequent prevention of breakthrough in the eculizimab treatment plan.

7.4 The P furthermore referred to D13 (page 1021, left column, 1st paragraph) and argued that D13 was teaching away from the presently claimed schedule since it suggested that the inhibition of C5 had to be cautiously balanced.

7.5 The P also noted that in the present context the decision T1979/09 cited by the OD (cited in the summons, point 2.23 of summons of 14. January 2014) was not applicable and referred to the corresponding arguments which had already been set out in his reply to the summons. The P furthermore referred to decision T1020/03 (point 72) in support of his arguments in favor of inventive step.

7.6 Before announcing a break, the CH asked the P if he had any further comments on the arguments which had been brought forth by the opponent. The P replied that the remaining documents cited by the opponent were regarded as less relevant so that he had no further comments.

8 After the break, the CH informed the P that the OD was of the opinion that the MR did not fulfil the requirements of Article 56 EPC. The claimed subject-matter was considered obvious over a combination of documents D5 (as CPA) and D6.

9 The CH opened the discussion on the AR1 and invited the P to indicate the basis under Article 123(2) EPC for the amendments.

9.1 The P referred to page 38 (lines 11-17) and Figure 8 as a basis for the amendments of the AR1.

10 After a break, the CH shared the opinion of the OD that the AR1 met the requirements of Articles 123(2), 123(3), 54 and 84 EPC as well as of Rule 80 EPC and opened the discussion on inventive step.

10.1 The P stated that the independent claims of the auxiliary request introduced both the parameter according to which complement breakthrough should be measured (namely cell lysis) and the threshold level which marks the efficient inhibition of breakthrough (namely less than 20%). The P argued that neither D5, let alone D6 (which did not even relate to complement breakthrough) did establish these parameters which allow the skilled person to efficiently evaluate the problem of breakthrough and the effect of counteracting measures.

10.2 Upon enquiry by the CH, the P explained that in the prior art breakthrough had rather been measured by means of urine color etc.

10.3 Before the CH announced a break, the first examiner (EX1) also noted that there was a contradiction between page 38, lines 11-17 which stated that two patients exhibited breakthrough and Figure 8 where 4 patients where observed to cross the 20% threshold transiently.

11 After the break, the P named further means of measuring complement breakthrough which had been used in the prior art, among them e.g. LDH and AST levels as well as EORTC QLC30 (quality of life) as disclosed in D6. The P emphasized that the method of the present application was superior to these measures since it provided a *direct measure* of complement breakthrough. With respect to the contradiction of page 38 and Figure 8, the P emphasized that the important conclusion of Figure 8 was that the long-term values for all patients are below 20%.

12 After a further break, the CH informed the P that the OD was of the opinion that the AR1 did not fulfill the requirements of Article 100(a) EPC in conjunction with Article 56 EPC

13 The P stated that he had no further requests or comments.

14 The C announced the decision of the OD to revoke the patent under Article 101(3)(b) for failure of the MR and the AR1 to comply with Article 100(a) EPC in conjunction with Article 56 EPC. The CH informed the P that the decision of the OD was open to appeal.

15 The CH closed the OP.

After deliberation of the opposition division,

- the chairman announced the following **decision**:

"The European patent is revoked."

Regarding the reasons for the decision, the chairman referred to:

Article 101(3)(b)EPC: Taking into consideration the amendments made by the proprietor of the patent during the opposition proceedings, the patent and the invention to which it relates do **not** meet the requirements of the Convention

The main request and auxiliary request do not meet the criteria of Article 56 EPC

The chairman **closed the oral proceedings** on 15.05.2014 at 14:15 hours.

Cilensek, Zoran
Chairman

Weikl, Martina
Minute Writer

Annex(es):

Annex 1

EP 05 71 2607.0 / 1720571
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Our Ref.: M2367 OPP(EP)

D14

Declaration of Leonard Bell, M.D.

1. I, Leonard Bell, M.D., a citizen of the United States, do reside at 59 Tumblebrook Road, Woodbridge, CT 06525. I am of the age of majority, and am aware that this Declaration is submitted in support of the European Patent EP 1 720 571 ("the Patent"), of which I am a co-inventor.

2. I am a founder and the present Chief Executive Officer of Alexion Pharmaceuticals, Inc. Prior to founding Alexion, I was an attending physician at Yale-New Haven Hospital and Assistant Professor of Medicine and Pathology at the Yale University School of Medicine, where I also received my M.D. degree. I remain on the faculty of the Yale University School of Medicine as an Adjunct Assistant Professor of Medicine and Pathology. I received my A.B. degree from Brown University.

3. Claim 1 of European Patent No. 1 720 571 is drawn to the use of the antagonist anti-C5 antibody eculizumab for treating a specific subgroup of patients afflicted with paroxysmal nocturnal hemoglobinuria (PNH). These patients are to be treated with eculizumab by intravenous infusion and under the following dosing schedule: (i) 600 mg each week for four weeks; (ii) 900 mg 1 week later; (iii) 900 mg on a biweekly basis; and, in the event of a breakthrough of complement activity, thereafter (iv) 900 mg every 12 days. Step (i) of this dosing schedule is a "loading" (or "induction") dose for eculizumab, whereas steps (ii), (iii), and (iv) are "maintenance" doses. Importantly, steps (i) and (ii) establish an initial, basal therapeutic level of complement inhibition that can be further maintained by step (iii), or step (iv) in the case of the subgroup of breakthrough patients recited in the claims. This schedule is described by, and exemplified in, the working examples of the Patent.

4. The dose recited in step (ii) – 900 mg of eculizumab administered to the PNH patient on week five – is especially important for the instantly-specified subgroup of breakthrough patients. This dose is important because, without it, the loading dose specified by step (i) does not provide an adequate baseline of

complement inhibition that can be maintained by the maintenance dose of step (iii) or (iv).

5. The dose specified by step (ii) was included in the PNH dosing schedule based on the results of clinical studies investigating, among other things, the pharmacokinetic and pharmacodynamic properties of eculizumab in human participants. In a subset of those studies, the dosing schedule administered to the participants consisted of a loading dose – 600 mg each week for five weeks – followed by a maintenance dose of 600 mg every two weeks. On that schedule, complement inhibition was inconsistently blocked in the participants. This observation indicated that the final 600 mg loading dose of eculizumab was insufficient to maintain a therapeutic level of complement inhibition for the two weeks prior to the start of the maintenance dosing schedule. As noted above, addition of the step (ii) dose of 900 mg, an increased dose relative to the insufficient final loading dose of 600 mg above, provides a trough level of eculizumab sufficient to achieve complete terminal complement inhibition that can be maintained by step (iii), or for patients who experience breakthrough, step (iv). Maintaining complement suppression in PNH patients is particularly important because, as the specification of the Patent states at paragraphs [0002] to [0005], failure to do so results in, among other pathologic sequelae, complement-mediated hemolysis and thrombosis, the latter of which can be fatal, and is the leading cause of death in patients with PNH.

6. The U.S. product insert for Soliris®, the tradename under which eculizumab is sold, exemplifies the importance of the second loading dose specified by step (ii) of claim 1. Namely, Point 2.1 of the product insert provides the recommended dosage regimen of eculizumab for PNH patients as: "600 mg weekly for the first 4 weeks, followed by 900 mg for the fifth dose 1 week later, then 900 mg every 2 weeks thereafter."

7. All statements made herein made on the basis of my own knowledge are true; all statements made herein on information and belief are believed to be true.

5/14/2014.
Date

Leonard Bell, M.D.

Annex 2

HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use Soliris safely and effectively. See full prescribing information for Soliris.

Soliris® (eculizumab)

Concentrated solution for intravenous infusion
Initial U.S. Approval: 2007

WARNING: SERIOUS MENINGOCOCCAL INFECTIONS

See full prescribing information for complete boxed warning

Life-threatening and fatal meningococcal infections have occurred in patients treated with Soliris. Meningococcal infection may become rapidly life-threatening or fatal if not recognized and treated early (5.1).

- **Comply with the most current Advisory Committee on Immunization Practices (ACIP) recommendations for meningococcal vaccination in patients with complement deficiencies.**
- **Immunize patients with a meningococcal vaccine at least 2 weeks prior to administering the first dose of Soliris, unless the risks of delaying Soliris therapy outweigh the risks of developing a meningococcal infection. (See *Serious Meningococcal Infections* (5.1) for additional guidance on the management of the risk of meningococcal infection.)**
- **Monitor patients for early signs of meningococcal infections, and evaluate immediately if infection is suspected.**

Soliris is available only through a restricted program under a Risk Evaluation and Mitigation Strategy (REMS). Under the Soliris REMS prescribers must enroll in the program (5.2). Enrollment in the Soliris REMS program and additional information are available by telephone: 1-888-SOLIRIS (1-888-765-4747).

RECENT MAJOR CHANGES

Box Warning	9/2011
Indications and Usage (1.2)	9/2011
Contraindications (4)	9/2011
Dosage and Administration (2.2)	9/2011
Warnings and Precautions (5.3, 5.4 and 5.6)	9/2011
Adverse Reactions (6.1)	9/2011
Use in Specific Populations (8.4)	9/2011
Clinical Studies (14.2)	9/2011
Patient Counseling Information (17)	9/2011

INDICATIONS AND USAGE

Soliris is a complement inhibitor indicated for:

- The treatment of patients with paroxysmal nocturnal hemoglobinuria (PNH) to reduce hemolysis (1.1).
- The treatment of patients with atypical hemolytic uremic syndrome (aHUS) to inhibit complement-mediated thrombotic microangiopathy (1.2).

The effectiveness of Soliris in aHUS is based on the effects on thrombotic microangiopathy (TMA) and renal function. Prospective clinical trials in additional patients are ongoing to confirm the benefit of Soliris in patients with aHUS.

Limitation of Use

Soliris is not indicated for the treatment of patients with Shiga toxin *E. coli* related hemolytic uremic syndrome (STEC-HUS).

DOSAGE AND ADMINISTRATION

Only administer as an intravenous infusion

PNH Dosage Regimen: (2.1)
aHUS Dosage Regimen: (2.2)

DOSAGE FORMS AND STRENGTHS

300 mg single-use vials each containing 30 mL of 10 mg/mL sterile, preservative-free solution (3).

CONTRAINDICATIONS

Soliris is contraindicated in:

- Patients with unresolved serious *Neisseria meningitidis* infection (4).
- Patients who are not currently vaccinated against *Neisseria meningitidis*, unless the risks of delaying Soliris treatment outweigh the risks of developing a meningococcal infection (5.1).

WARNINGS AND PRECAUTIONS

- Discontinue Soliris in patients who are being treated for serious meningococcal infections.
- Use caution when administering Soliris to patients with any other systemic infection (5.2).

ADVERSE REACTIONS

The most frequently reported adverse reactions in the PNH randomized trial (≥10% overall and greater than placebo) are: headache, nasopharyngitis, back pain, and nausea (6.1).

The most frequently reported adverse reactions in aHUS single arm prospective trials (≥15% combined per patient incidence) are: hypertension, upper respiratory tract infection, diarrhea, headache, anemia, vomiting, nausea, urinary tract infection, and leukopenia (6.1).

To report SUSPECTED ADVERSE REACTIONS, contact Alexion Pharmaceuticals, Inc. at 1-888-SOLIRIS (1-888-765-4747) or FDA at 1-800-FDA-1088 or www.fda.gov/medwatch.

USE IN SPECIFIC POPULATIONS

Pregnancy: Based on animal data, Soliris may cause fetal harm (8.1).

Nursing Mothers: Caution should be exercised when administered to a nursing woman (8.3).

Pediatric Use: PNH: safety and effectiveness not established. aHUS: safety and effectiveness similar to adult patients (8.4).

See 17 PATIENT COUNSELING INFORMATION and the FDA-approved Medication Guide.

Revised: 09/2011

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***Sections or subsections omitted from the full prescribing information are not listed.**

FULL PRESCRIBING INFORMATION

WARNING: SERIOUS MENINGOCOCCAL INFECTIONS

Life-threatening and fatal meningococcal infections have occurred in patients treated with Soliris. Meningococcal infection may become rapidly life-threatening or fatal if not recognized and treated early (5.1).

- Comply with the most current Advisory Committee on Immunization Practices (ACIP) recommendations for meningococcal vaccination in patients with complement deficiencies.
- Immunize patients with a meningococcal vaccine at least 2 weeks prior to administering the first dose of Soliris, unless the risks of delaying Soliris therapy outweigh the risk of developing a meningococcal infection. (See *Serious Meningococcal Infections (5.1)* for additional guidance on the management of the risk of meningococcal infection.)
- Monitor patients for early signs of meningococcal infections and evaluate immediately if infection is suspected.

Soliris is available only through a restricted program under a Risk Evaluation and Mitigation Strategy (REMS). Under the Soliris REMS, prescribers must enroll in the program (5.2). Enrollment in the Soliris REMS program and additional information are available by telephone: 1-888-SOLIRIS (1-888-765-4747).

1 INDICATIONS AND USAGE

1.1 Paroxysmal Nocturnal Hemoglobinuria (PNH)

Soliris is indicated for the treatment of patients with paroxysmal nocturnal hemoglobinuria (PNH) to reduce hemolysis.

1.2 Atypical Hemolytic Uremic Syndrome (aHUS)

Soliris is indicated for the treatment of patients with atypical hemolytic uremic syndrome (aHUS) to inhibit complement-mediated thrombotic microangiopathy.

The effectiveness of Soliris in aHUS is based on the effects on thrombotic microangiopathy (TMA) and renal function. Prospective clinical trials in additional patients are ongoing to confirm the benefit of Soliris in patients with aHUS.

Limitation of Use

Soliris is not indicated for the treatment of patients with Shiga toxin *E. coli* related hemolytic uremic syndrome (STEC-HUS).

2 DOSAGE AND ADMINISTRATION

Healthcare professionals who prescribe Soliris must enroll in the Soliris REMS. [*see Warnings and Precautions (5.2)*].

Vaccinate patients according to current ACIP guidelines to reduce the risk of serious infection. [*see Warnings and Precautions (5.1) and (5.2)*].

Only administer as an intravenous infusion.

2.1 Recommended Dosage Regimen - PNH

Soliris therapy consists of:

- 600 mg weekly for the first 4 weeks, followed by
- 900 mg for the fifth dose 1 week later, then
- 900 mg every 2 weeks thereafter.

Soliris should be administered at the recommended dosage regimen time points, or within two days of these time points *[see Warnings and Precautions (5.6)]*.

2.2 Recommended Dosage Regimen - aHUS

For patients 18 years of age and older, Soliris therapy consists of:

- 900 mg weekly for the first 4 weeks, followed by
- 1200 mg for the fifth dose 1 week later, then
- 1200 mg every 2 weeks thereafter.

For patients less than 18 years of age, administer Soliris based upon body weight, according to the following schedule (Table 1):

Table 1: Dosing recommendations in patients less than 18 years of age

Patient Body Weight	Induction	Maintenance
40 kg and over	900 mg weekly x 4 doses	1200 mg at week 5; then 1200 mg every 2 weeks
30 kg to less than 40 kg	600 mg weekly x 2 doses	900 mg at week 3; then 900 mg every 2 weeks
20 kg to less than 30 kg	600 mg weekly x 2 doses	600 mg at week 3; then 600 mg every 2 weeks
10 kg to less than 20 kg	600 mg weekly x 1 dose	300 mg at week 2; then 300 mg every 2 weeks
5 kg to less than 10 kg	300 mg weekly x 1 dose	300 mg at week 2; then 300 mg every 3 weeks

Soliris should be administered at the recommended dosage regimen time points, or within two days of these time points.

Supplemental dosing of Soliris is required in the setting of concomitant support with PE/PI (plasmapheresis or plasma exchange; or fresh frozen plasma infusion) (Table 2).

Table 2: Supplemental dose of Soliris after PE/PI

Type of Intervention	Most Recent Soliris Dose	Supplemental Soliris Dose With Each PE/PI Intervention	Timing of Supplemental Soliris Dose
Plasmapheresis or plasma exchange	300 mg	300 mg per each plasmapheresis or plasma exchange session	Within 60 minutes after each plasmapheresis or plasma exchange
	600 mg or more	600 mg per each plasmapheresis or plasma exchange session	
Fresh frozen plasma infusion	300 mg or more	300 mg per each unit of fresh frozen plasma	60 minutes prior to each 1 unit of fresh frozen plasma infusion

2.3 Preparation and Administration

Soliris must be diluted to a final admixture concentration of 5 mg/mL using the following steps:

- Withdraw the required amount of Soliris from the vial into a sterile syringe.
- Transfer the recommended dose to an infusion bag.
- Dilute Soliris to a final concentration of 5 mg/mL by adding the appropriate amount (equal volume of diluent to drug volume) of 0.9% Sodium Chloride Injection, USP; 0.45% Sodium Chloride Injection, USP; 5% Dextrose in Water Injection, USP; or Ringer's Injection, USP to the infusion bag.

The final admixed Soliris 5 mg/mL infusion volume is 60 mL for 300 mg doses, 120 mL for 600 mg doses, 180 mL for 900 mg doses or 240 mL for 1200 mg doses (Table 3).

Table 3: Preparation and Reconstitution of Soliris

Soliris Dose	Diluent Volume	Final Volume
300 mg	30 mL	60 mL
600 mg	60 mL	120 mL
900 mg	90 mL	180 mL
1200 mg	120 mL	240 mL

Gently invert the infusion bag containing the diluted Soliris solution to ensure thorough mixing of the product and diluent. Discard any unused portion left in a vial, as the product contains no preservatives.

Prior to administration, the admixture should be allowed to adjust to room temperature [18°-25° C, 64-77° F]. The admixture must not be heated in a microwave or with any heat source other than ambient air temperature. The Soliris admixture should be inspected visually for particulate matter and discoloration prior to administration.

2.4 Administration

Do Not Administer As An Intravenous Push or Bolus Injection

The Soliris admixture should be administered by intravenous infusion over 35 minutes via gravity feed, a syringe-type pump, or an infusion pump. Admixed solutions of Soliris are stable for 24 hours at 2-8° C (36-46° F) and at room temperature.

If an adverse reaction occurs during the administration of Soliris, the infusion may be slowed or stopped at the discretion of the physician. If the infusion is slowed, the total infusion time should not exceed two hours. Monitor the patient for at least one hour following completion of the infusion for signs or symptoms of an infusion reaction.

3 DOSAGE FORMS AND STRENGTHS

Soliris is supplied as 300 mg single-use vials each containing 30 mL of 10 mg/mL sterile, preservative-free eculizumab solution.

4 CONTRAINDICATIONS

Soliris is contraindicated in:

- Patients with unresolved serious *Neisseria meningitidis* infection
- Patients who are not currently vaccinated against *Neisseria meningitidis*, unless the risks of delaying Soliris treatment outweigh the risks of developing a meningococcal infection *[see Warnings and Precautions (5.1)]*.

5 WARNINGS AND PRECAUTIONS

5.1 Serious Meningococcal Infections

The use of Soliris increases a patient's susceptibility to serious meningococcal infections (septicemia and/or meningitis). Life-threatening and fatal meningococcal infections have occurred in patients treated with Soliris.

Administer a polyvalent meningococcal vaccine according to the most current Advisory Committee on Immunization Practices (ACIP) recommendations for patients with complement deficiencies. Revaccinate patients in accordance with ACIP recommendations, considering the duration of Soliris therapy.

Vaccinate patients without a history of meningococcal vaccination at least 2 weeks prior to receiving the first dose of Soliris. If urgent Soliris therapy is indicated in an unvaccinated patient, administer the meningococcal vaccine as soon as possible. In clinical studies, 33/67 patients with aHUS were treated with Soliris less than 2 weeks after meningococcal vaccination and 31 of these 33 patients received antibiotics for prophylaxis of meningococcal infection until at least 2 weeks after meningococcal vaccination. The benefits and risks of antibiotic prophylaxis for prevention of meningococcal infections in patients receiving Soliris have not been established.

Vaccination reduces, but does not eliminate, the risk of meningococcal infections. In clinical studies, 2 out of 196 PNH patients developed serious meningococcal infections while receiving treatment with Soliris; both had been vaccinated *[see Adverse Reactions (6.1)]*. In clinical studies among non-PNH patients, meningococcal meningitis occurred in one unvaccinated patient. In addition, a previously vaccinated patient with aHUS developed meningococcal sepsis during the post-study follow-up period *[see Adverse Reactions (6.1)]*.

Closely monitor patients for early signs and symptoms of meningococcal infection and evaluate patients immediately if an infection is suspected. Meningococcal infection may become rapidly life-threatening or fatal if not recognized and treated early. Discontinue Soliris in patients who are undergoing treatment for serious meningococcal infections.

5.2 Soliris REMS

Because of the risk of meningococcal infections, Soliris is available only through a restricted program under a Risk Evaluation and Mitigation Strategy (REMS). Under the Soliris REMS, prescribers must enroll in the program.

Prescribers must counsel patients about the risk of meningococcal infection, provide the patients with the REMS educational materials, and ensure patients are vaccinated with a meningococcal vaccine.

Enrollment in the Soliris REMS program and additional information are available by telephone: 1-888-SOLIRIS (1-888-765-4747).

5.3 Other Infections

Soliris blocks terminal complement activation; therefore patients may have increased susceptibility to infections, especially with encapsulated bacteria. Children treated with Soliris may be at increased risk of developing serious infections due to *Streptococcus pneumoniae* and *Haemophilus influenza* type b (Hib). Administer vaccinations for the prevention of *Streptococcus pneumoniae* and *Haemophilus influenza* type b (Hib) infections according to ACIP guidelines. Use caution when administering Soliris to patients with any systemic infection.

5.4 Monitoring After Soliris Discontinuation

Treatment Discontinuation for PNH

Monitor patients after discontinuing Soliris for at least 8 weeks to detect hemolysis.

Treatment Discontinuation for aHUS

After discontinuing Soliris, monitor patients with aHUS for signs and symptoms of thrombotic microangiopathy (TMA) complications for at least 12 weeks. In aHUS clinical studies, 18 patients (5 in the prospective studies) discontinued Soliris treatment. TMA complications occurred following a missed dose in 5 patients, and Soliris was reinitiated in 4 of these 5 patients.

Clinical signs and symptoms of TMA include changes in mental status, seizures, angina, dyspnea, or thrombosis. In addition, the following changes in laboratory parameters may identify a TMA complication: occurrence of two, or repeated measurement of any one of the following: a decrease in platelet count by 25% or more compared to baseline or the peak platelet count during Soliris treatment; an increase in serum creatinine by 25% or more compared to baseline or nadir during Soliris treatment; or, an increase in serum LDH by 25% or more over baseline or nadir during Soliris treatment.

If TMA complications occur after Soliris discontinuation, consider reinstitution of Soliris treatment, plasma therapy [plasmapheresis, plasma exchange, or fresh frozen plasma infusion (PE/PI)], or appropriate organ-specific supportive measures.

5.5 Thrombosis Prevention and Management

The effect of withdrawal of anticoagulant therapy during Soliris treatment has not been established. Therefore, treatment with Soliris should not after anticoagulant management.

5.6 Laboratory Monitoring

PNH

Serum LDH levels increase during hemolysis and may assist in monitoring Soliris effects, including the response to discontinuation of therapy. In clinical studies, six patients achieved a reduction in serum LDH levels only after a decrease in the Soliris dosing interval from 14 to 12 days. All other patients achieved a reduction in serum LDH levels with the 14 day dosing interval *[see Clinical Pharmacology (12.2) and Clinical Studies (14.1)]*.

aHUS

Early signs of thrombotic microangiopathy (TMA) include a decrease in platelet count, and

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increases in serum LDH and creatinine levels. Follow patients for signs of TMA by monitoring serial platelet counts, serum LDH, and creatinine during Soliris therapy and following discontinuation of Soliris.

5.7 Infusion Reactions

As with all protein products, administration of Soliris may result in infusion reactions, including anaphylaxis or other hypersensitivity reactions. In clinical trials, no patients experienced an infusion reaction which required discontinuation of Soliris. Interrupt Soliris infusion and institute appropriate supportive measures if signs of cardiovascular instability or respiratory compromise occur.

6 ADVERSE REACTIONS

6.1 Clinical Trial Experience

Meningococcal infections are the most important adverse reactions experienced by patients receiving Soliris. In PNH clinical studies, two patients experienced meningococcal sepsis. Both patients had previously received a meningococcal vaccine. In clinical studies among patients without PNH, meningococcal meningitis occurred in one unvaccinated patient. Meningococcal sepsis occurred in one previously vaccinated patient enrolled in the retrospective aHUS study during the post-study follow-up period [*see Warnings and Precautions (5.1)*].

PNH

The data described below reflect exposure to Soliris in 196 adult patients with PNH, age 18-85, of whom 55% were female. All had signs or symptoms of intravascular hemolysis. Soliris was studied in a placebo-controlled clinical study (in which 43 patients received Soliris and 44, placebo); a single arm clinical study and a long term extension study. 182 patients were exposed for greater than one year. All patients received the recommended Soliris dose regimen.

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice. Table 4 summarizes the adverse reactions that occurred at a numerically higher rate in the Soliris group than the placebo group and at a rate of 5% or more among patients treated with Soliris.

Table 4: Adverse Reactions Reported In 5% Or More Of Soliris Treated Patients And Greater Than Placebo In The Controlled Clinical Study

Reaction	Soliris N = 43 N (%)	Placebo N = 44 N (%)
Headache	19 (44)	12 (27)
Nasopharyngitis	10 (23)	8 (18)
Back pain	8 (19)	4 (9)
Nausea	7 (16)	5 (11)
Fatigue	5 (12)	1 (2)
Cough	5 (12)	4 (9)
Herpes simplex infections	3 (7)	0
Sinusitis	3 (7)	0
Respiratory tract infection	3 (7)	1 (2)
Constipation	3 (7)	2 (5)
Myalgia	3 (7)	1 (2)
Pain in extremity	3 (7)	1 (2)
Influenza-like illness	2 (5)	1 (2)

In the placebo-controlled clinical study, serious adverse reactions occurred among 4 (9%) patients receiving Soliris and 9 (21%) patients receiving placebo. The serious reactions included infections and progression of PNH. No deaths occurred in the study and no patients receiving Soliris experienced a thrombotic event; one thrombotic event occurred in a patient receiving placebo.

Among 193 patients with PNH treated with Soliris in the single arm, clinical study or the follow-up study, the adverse reactions were similar to those reported in the placebo-controlled clinical study. Serious adverse reactions occurred among 16% of the patients in these studies. The most common serious adverse reactions were: viral infection (2%), headache (2%), anemia (2%), and pyrexia (2%).

aHUS

The safety of Soliris therapy in patients with aHUS was evaluated in two prospective, single-arm studies (aHUS Studies 1 and 2) and one retrospective study (aHUS Study 3). The data described below were derived from 37 adult and adolescent patients with aHUS enrolled in aHUS Study 1 and aHUS Study 2. All patients received the recommended dosage of Soliris. Median exposure was 38 weeks (range: 2-64 weeks).

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice. Table 5 summarizes all adverse events reported in at least 10% of patients in aHUS Studies 1 and 2 combined.

Table 5: Per Patient Incidence of Adverse Events in Adult and Adolescent Patients Enrolled in aHUS Study 1 and aHUS Study 2 Separately and in Total

MedDRA ver. 11.0	Number (%) of Patients Study 1 (n=17)	Study 2 (n=20)	Total (n=37)
Vascular Disorders			
Hypertension[a]	8(47)	5 (25)	13 (35)
Infections and Infestations			
Upper respiratory tract infection[b]	5 (29)	8 (40)	13(35)
Urinary tract infection	4 (24)	2 (10)	6 (16)
Gastrointestinal Disorders			
Diarrhea	6 (35)	6 (30)	12 (32)
Vomiting	5 (29)	3 (15)	8 (22)
Nausea	4 (24)	3 (15)	7 (19)
Abdominal pain	0	4 (20)	4 (11)
Nervous System Disorders			
Headache	7 (41)	4 (20)	11 (30)
Blood and Lymphatic System Disorders			
Anemia	6 (35)	3 (15)	9 (24)
Leukopenia	4 (24)	2 (10)	6 (16)
Psychiatric Disorders			
Insomnia	4 (24)	1 (5)	5 (14)
Respiratory, Thoracic and Mediastinal Disorders			
Cough	2 (12)	3 (15)	5 (14)
Pharyngolaryngeal pain	1 (6)	4 (20)	5 (14)
General Disorders and Administration Site Conditions			
Fatigue	3 (18)	1 (5)	4 (11)
Peripheral edema	3 (18)	1 (5)	4 (11)
Pyrexia	3 (18)	1 (5)	4 (11)
Ear and Labyrinth Disorders			
Vertigo	1 (6)	3 (15)	4 (11)
Musculoskeletal and Connective Tissue Disorders			
Pain in extremity	1 (6)	3 (15)	4 (11)

[a] includes the preferred terms hypertension, accelerated hypertension, and malignant hypertension.

[b] includes the preferred terms upper respiratory tract infection and nasopharyngitis.

In aHUS Studies 1 and 2 combined, 54% (20/37) of patients experienced a serious adverse event (SAE). The most commonly reported SAEs were hypertension (16%) and infections (14%). One patient discontinued Soliris due to adverse events deemed unrelated to Soliris.

Analysis of retrospectively collected adverse event data from pediatric and adult patients enrolled in aHUS Study 3 (N=30) revealed a safety profile that was similar to that which was observed in the two prospective studies. aHUS Study 3 included 19 pediatric patients less than 18 years of age. Overall, the safety of Soliris in pediatric patients with aHUS enrolled in Study 3 appeared similar to that observed in adult patients. The most common (≥15%) adverse events occurring in pediatric patients are presented in Table 6.

Table 6: Adverse Reactions Occurring in at Least 15% of Patients Less than 18 Years of Age Enrolled in aHUS Study 3

MedDRA ver. 11.0	Number (%) of Patients < 2 yrs (n=5)	2 to < 12 yrs (n=10)	12 to <18 yrs (n=4)	Total (n=19)
General Disorders and Administration Site Conditions				
Pyrexia	4 (80)	4 (60)	1 (25)	9 (47)
Gastrointestinal Disorders				
Diarrhea	1 (20)	4 (40)	1 (25)	6 (32)
Vomiting	2 (40)	1 (10)	1 (25)	4 (21)
Infections and Infestations				
Upper respiratory tract infection[a]	2 (40)	3 (30)	1 (25)	6 (32)
Respiratory, Thoracic and Mediastinal Disorders				
Cough	3 (60)	2 (20)	0 (0)	5 (26)
Nasal congestion	2 (40)	2 (20)	0 (0)	4 (21)
Cardiac Disorders				
Tachycardia	2 (40)	2 (20)	0 (0)	4 (21)

[a] includes the preferred terms upper respiratory tract infection and nasopharyngitis.

6.2 Immunogenicity

As with all proteins there is a potential for immunogenicity. The immunogenicity of Soliris has been evaluated using two different immunoassays for the detection of anti-eculizumab antibodies: a direct enzyme-linked immunosorbent assay (ELISA) using the Fab fragment of eculizumab as target was used for the PNH indication; and an electro-chemiluminescence (ECL) bridging assay using the eculizumab whole molecular as target was used for the aHUS indication. Low titers of antibodies to Soliris were detected in 3/196 (2%) of all PNH patients treated with Soliris by the ELISA assay. In patients with aHUS treated with Soliris, antibodies to Soliris were detected in 1/37 (2.7%) by the ECL assay. An ECL based neutralizing HAHA assay with a low sensitivity of 2 mcg/mL was performed to detect neutralizing antibodies for the 37 patients with aHUS. No neutralizing activity to Soliris was detected in patients with aHUS treated with Soliris. No apparent correlation of antibody development to clinical response was observed in both indications. The immunogenicity data reflect the percentage of patients whose test results were considered positive for antibodies to Soliris in an ELISA based assay and/or an ECL based assay are highly dependent on the sensitivity and specificity of the assay used. Additionally, the observed incidence of antibody positivity in the assay may be influenced by several factors including sample handling, timing of sample collection, concomitant medications and underlying disease. For these reasons, comparison of the incidence of antibodies to Soliris with the incidence of antibodies to other products may be misleading.

6.3 Postmarketing Experience

Cases of serious or fatal meningococcal infections have been reported.

7 DRUG INTERACTIONS

Drug interaction studies have not been performed with Soliris.

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8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Pregnancy Category C:

There are no adequate and well-controlled studies of Soliris in pregnant women. Soliris, a recombinant IgG molecule (humanized anti-C5 antibody), is expected to cross the placenta. Animal studies using a mouse analogue of the Soliris molecule (murine anti-C5 antibody) showed increased rates of developmental abnormalities and an increased rate of dead and moribund offspring at doses 2-8 times the human dose. Soliris should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Animal reproduction studies were conducted in mice using doses of a murine anti-C5 antibody that approximated 2-4 times (low dose) and 4-8 times (high dose) the recommended human Soliris dose, based on a body weight comparison. When animal exposure to the antibody occurred in the time period from before mating until early gestation, no decrease in fertility or reproductive performance was observed. When maternal exposure to the antibody occurred during organogenesis, two cases of retinal dysplasia and one case of umbilical hernia were observed among 230 offspring born to mothers exposed to the higher antibody dose; however, the exposure did not increase fetal loss or neonatal death. When maternal exposure to the antibody occurred in the time period from implantation through weaning, a higher number of male offspring became moribund or died (1/25 controls, 2/25 low dose group, 5/25 high dose group). Surviving offspring had normal development and reproductive function.

8.3 Nursing Mothers

It is not known whether Soliris is excreted into human milk. IgG is excreted in human milk, so it is expected that Soliris will be present in human milk. However, published data suggest that antibodies in human milk do not enter the neonatal and infant circulation in substantial amounts. Caution should be exercised when Soliris is administered to a nursing woman. The unknown risks to the infant from gastrointestinal or limited systemic exposure to Soliris should be weighed against the known benefits of human milk feeding.

8.4 Pediatric Use

The safety and effectiveness of Soliris for the treatment of PNH in pediatric patients below the age of 18 years have not been established.

Three clinical studies assessing the safety and effectiveness of Soliris for the treatment of aHUS included a total of 25 pediatric patients (ages 2 months to 17 years). The safety and effectiveness of Soliris for the treatment of aHUS appear similar in pediatric and adult patients *[see Dosage and Administration (2.2), Adverse Reactions (6.1), and Clinical Studies (14.2)]*.

Administer vaccinations for the prevention of infection due to *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenza* type b (Hib) according to ACIP guidelines *[see Warnings and Precautions (5.1, 5.2)]*.

8.5 Geriatric Use

Sixteen patients 65 years of age or older (15 with PNH and 1 with aHUS) were treated with Soliris. Although there were no apparent age-related differences observed in these studies, the number of patients aged 65 and over is not sufficient to determine whether they respond differently from younger patients.

10 OVERDOSAGE

No cases of Soliris overdose have been reported during clinical studies.

11 DESCRIPTION

Soliris, a complement inhibitor, is a formulation of eculizumab which is a recombinant humanized monoclonal IgG2/4$_K$ antibody produced by murine myeloma cell culture and purified by standard bioprocess technology. Eculizumab contains human constant regions from human IgG2 sequences and human IgG4 sequences and murine complementarity-determining regions grafted onto the human framework light- and heavy-chain variable regions. Eculizumab is composed of two 448 amino acid heavy chains and two 214 amino acid light chains and has a molecular weight of approximately 148 kDa.

Soliris is a sterile, clear, colorless, preservative-free 10 mg/mL solution for intravenous infusion and is supplied in 30-mL single-use vials. The product is formulated at pH 7 and each vial contains 300 mg of eculizumab, 13.8 mg sodium phosphate monobasic, 53.4 mg sodium phosphate dibasic, 263.1 mg sodium chloride, 6.6 mg polysorbate 80 (vegetable origin) and Water for Injection, USP.

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

Eculizumab, the active ingredient in Soliris, is a monoclonal antibody that specifically binds to the complement protein C5 with high affinity, thereby inhibiting its cleavage to C5a and C5b and preventing the generation of the terminal complement complex C5b-9. Soliris inhibits terminal complement mediated intravascular hemolysis in PNH patients and complement-mediated thrombotic microangiopathy (TMA) in patients with aHUS.

A genetic mutation in patients with PNH leads to the generation of populations of abnormal RBCs (known as PNH cells) that are deficient in terminal complement inhibitors, rendering PNH RBCs sensitive to persistent terminal complement-mediated destruction. The destruction and loss of these PNH cells (intravascular hemolysis) results in low RBC counts (anemia), and also fatigue, difficulty in functioning, pain, dark urine, shortness of breath, and blood clots.

In aHUS, impairment in the regulation of complement activity leads to uncontrolled terminal complement activation, resulting in platelet activation, endothelial cell damage and thrombotic microangiopathy.

12.2 Pharmacodynamics

In the PNH placebo-controlled clinical study, Soliris when administered as recommended reduced hemolysis as shown by the reduction of serum LDH levels from 2200 ± 1034 U/L (mean ± SD) at baseline to 700 ± 388 U/L by week one and maintained the effect through the end of the study at week 26 (327 ± 433 U/L). In the single arm clinical study, Soliris maintained this effect through 52 weeks *[see Clinical Studies (14)]*.

12.3. Pharmacokinetics

A population PK analysis with a standard 1-compartmental model was conducted on the multiple dose PK data from 40 PNH patients receiving the recommended Soliris regimen *[see Dosage and Administration (2.1)]*. In this model, the clearance of Soliris for a typical PNH patient weighing 70 kg was 22 mL/hr and the volume of distribution was 7.7 L. The half-life was 272 ± 82 hrs (mean ± SD). The mean observed peak and trough serum concentrations of Soliris by week 26 were 194 ± 76 mcg/mL and 97 ± 60 mcg/mL, respectively.

A second population PK analysis with a standard 1 compartmental model was conducted on the multiple dose PK data from 57 aHUS patients receiving the recommended Soliris regimen in studies 1, 2 and 3. In this model, the clearance of Soliris for a typical aHUS patient weighing 70 kg was 14.6 mL/hr and the volume of distribution was 6.14 L. The elimination half-life was 291 h (approximately 12.1 days).

The clearance and half-life of eculizumab were also evaluated during plasma exchange interventions. Plasma exchange increased the clearance of eculizumab to 3660 mL/hr and reduced the half-life to 1.26 hours. Supplemental dosing is recommended when Soliris is administered to aHUS patients receiving plasma infusion or exchange *[see Recommended Dosage Regimen (2.1)]*.

Dedicated studies have not been conducted to evaluate the PK of Soliris in special patient populations identified by gender, race, age (pediatric or geriatric), or the presence of renal or hepatic impairment. Pediatric and adolescent patients (less than 18 years of age) and patients with renal impairment were included in the aHUS clinical studies *[see Clinical Studies (14)]*. Population PK analysis showed age, gender, race, and renal function do not influence the PK of eculizumab.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

Long-term animal carcinogenicity studies of eculizumab have not been conducted.

Genotoxicity studies have not been conducted with eculizumab.

Effects of eculizumab upon fertility have not been studied in animals. Intravenous injections of male and female mice with a murine anti-C5 antibody at up to 4-8 times the equivalent of the clinical dose of Soliris had no adverse effects on mating or fertility.

14 CLINICAL STUDIES

14.1 PNH

The safety and efficacy of Soliris in PNH patients with hemolysis were assessed in a randomized, double-blind, placebo-controlled 26 week study (Study 1); PNH patients were also treated with Soliris in a single arm 52 week study (Study 2); and in a long term extension study. Patients received meningococcal vaccination prior to receipt of Soliris. In all studies, the dose of Soliris was 600 mg study drug every 7 ± 2 days for 4 weeks, followed by 900 mg 7 ± 2 days later, then 900 mg every 14 ± 2 days for the study duration. Soliris was administered as an intravenous infusion over 25 - 45 minutes.

Study 1:

PNH patients with at least four transfusions in the prior 12 months, flow cytometric confirmation of at least 10% PNH cells and platelet counts of at least 100,000/microliter were randomized to either Soliris (n = 43) or placebo (n = 44). Prior to randomization, all patients underwent an initial observation period to confirm the need for RBC transfusion and to identify the hemoglobin concentration (the "set-point") which would define each patient's hemoglobin stabilization and transfusion outcomes. The hemoglobin set-point was less than or equal to 9 g/dL in patients with symptoms and was less than or equal to 7 g/dL in patients without symptoms. Endpoints related to hemolysis included the numbers of patients achieving hemoglobin stabilization, the number of RBC units transfused, fatigue, and health-related quality of life. To achieve a designation of hemoglobin stabilization, a patient had to maintain a hemoglobin concentration above the hemoglobin set-point and avoid any RBC transfusion for the entire 26 week period. Hemolysis was monitored mainly by the measurement of serum LDH levels, and the proportion of PNH RBCs was monitored by flow cytometry. Patients receiving anticoagulants and systemic corticosteroids at baseline continued these medications.

Major baseline characteristics were balanced (see Table 7).

Table 7: PNH Study 1 Patient Baseline Characteristics

Parameter	Study 1 Placebo N = 44	Study 1 Soliris N = 43
Mean age (SD)	38 (13)	42 (16)
Gender - female (%)	29 (66)	23 (54)
History of aplastic anemia or myelodysplastic syndrome (%)	12 (27)	8 (19)
Patients with history of thrombosis (events)	8 (11)	9 (16)
Concomitant anticoagulants (%)	20 (46)	24 (56)
Concomitant steroids/immunosuppressant treatments (%)	16 (36)	14 (33)
Packed RBC units transfused per patient in previous 12 months (median (Q1,Q3))	17 (14, 25)	18 (12, 24)
Mean Hgb level (g/dL) at setpoint (SD)	8 (1)	8 (1)
Pre-treatment LDH levels (median, U/L)	2,234	2,032
Free hemoglobin at baseline (median, mg/dL)	46	41

Patients treated with Soliris had significantly reduced ($p < 0.001$) hemolysis resulting in improvements in anemia as indicated by increased hemoglobin stabilization and reduced need for RBC transfusions compared to placebo treated patients (see Table 8). These effects were seen among patients within each of the three pre-study RBC transfusion strata (4 - 14 units; 15 - 25 units; > 25 units). After 3 weeks of Soliris treatment, patients reported less fatigue and improved health-related quality of life. Because of the study sample size and duration, the effects of Soliris on thrombotic events could not be determined.

Table 8: PNH Study 1 Results

	Placebo N = 44	Soliris N = 43
Percentage of patients with stabilized hemoglobin levels	0	49
Packed RBC units transfused per patient (median) (range)	10 (2 - 21)	0 (0 - 16)
Transfusion avoidance (%)	0	51
LDH levels at end of study (median, U/L)	2,167	239
Free hemoglobin at end of study (median, mg/dL)	62	5

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Study 2 and Extension Study:
PNH patients with at least one transfusion in the prior 24 months and at least 30,000 platelets/microliter received Soliris over a 52-week period. Concomitant medications included anti-thrombotic agents in 63% of the patients and systemic corticosteroids in 40% of the patients. Overall, 96 of the 97 enrolled patients completed the study (one patient died following a thrombotic event). A reduction in intravascular hemolysis as measured by serum LDH levels was sustained for the treatment period and resulted in a reduced need for RBC transfusion and less fatigue. 187 Soliris-treated PNH patients were enrolled in a long term extension study. All patients sustained a reduction in intravascular hemolysis over a total Soliris exposure time ranging from 10 to 54 months. There were fewer thrombotic events with Soliris treatment than during the same period of time prior to treatment. However, the majority of patients received concomitant anticoagulants; the effects of anticoagulant withdrawal during Soliris therapy was not studied [*see Warnings and Precautions (5.4)*].

14.2 aHUS

Three single-arm studies [(two prospective (aHUS Studies 1 and 2) and one retrospective (aHUS Study 3)] evaluated the safety and efficacy of Soliris for the treatment of aHUS. Patients with aHUS received meningococcal vaccination prior to receipt of Soliris or received prophylactic treatment with antibiotics until 2 weeks after vaccination. In all studies, the dose of Soliris in adult and adolescent patients was 900 mg every 7 ± 2 days for 4 weeks, followed by 1200 mg 7 ± 2 days later, then 1200 mg every 14 ± 2 days thereafter. The dosage regimen for pediatric patients weighing less than 40 kg enrolled in aHUS Study 3 was based on body weight [*see Recommended Dosage Regimen (2.2)*]. Efficacy evaluations were based on thrombotic microangiopathy (TMA) endpoints.

Endpoints related to TMA included the following:

- platelet count change from baseline
- hematologic normalization *(maintenance of normal platelet counts and LDH levels for at least four weeks)*
- complete TMA response *(hematologic normalization plus at least a 25% reduction in serum creatinine for a minimum of four weeks)*
- TMA-event free status *(absence for at least 12 weeks of a decrease in platelet count of >25% from baseline, plasma exchange or plasma infusion, and new dialysis requirement)*
- Daily TMA intervention rate *(defined as the number of plasma exchange or plasma infusion interventions and the number of new dialyses required per patient per day)*.

aHUS Resistant to PE/PI (aHUS Study 1)

aHUS Study 1 enrolled patients who displayed signs of thrombotic microangiopathy (TMA) despite receiving at least four PE/PI treatments the week prior to screening. One patient had no PE/PI the week prior to screening because of PE/PI intolerance. In order to qualify for enrollment, patients were required to have a platelet count ≤150 x 10^9/L, evidence of hemolysis such as an elevation in serum LDH, and serum creatinine above the upper limits of normal, without the need for chronic dialysis. The median patient age was 28 (range: 17 to 68 years). Patients enrolled in aHUS Study 1 had an ADAMTS-13 level above 5%. Seventy-six percent of patients had an identified complement regulatory factor mutation or auto-antibody. Table 9 summarizes the key baseline clinical and disease-related characteristics of patients enrolled in aHUS Study 1.

Table 9: Baseline Characteristics of Patients Enrolled in aHUS Study 1

Parameter	aHUS Study 1 N = 17
Time from aHUS diagnosis until screening in months, median (min, max)	10 (0.26, 236)
Time from current clinical TMA manifestation until screening in months, median (min, max)	<1 (<1, 4)
Baseline platelet count (× 10^9/L), median (range)	118 (62, 161)
Baseline LDH (U/L), median (range)	269 (134, 634)

Patients in aHUS Study 1 received Soliris for a minimum of 26 weeks. After completion of the initial 26-week treatment period, most patients continued to receive Soliris by enrolling into an extension study. In aHUS Study 1, the median duration of Soliris therapy was approximately 38 weeks (range: 2 weeks to 64 weeks).

Reduction in terminal complement activity and an increase in platelet count relative to baseline were observed after commencement of Soliris. Soliris reduced signs of complement-mediated TMA activity, as shown by an increase in mean platelet counts from baseline to 26 weeks. In aHUS Study 1, mean platelet count increased from 109 ± 32 x10^9/L at baseline to 169 ± 72 x10^9/L by one week; this effect was maintained through 26 weeks (mean platelet count at week 26: 210 ± 68 x10^9/L). Table 10 summarizes the efficacy results for aHUS Study 1.

Table 10: Efficacy Results for aHUS Study 1 in PE/PI-Resistant aHUS

Efficacy Parameter	aHUS Study 1 N=17
Change in platelet count from baseline through week 26 (× 10^9/L), least square mean (95% CI)[1]	73 (40-105)
Hematologic normalization[2], n (%) Median Duration of hematologic normalization, weeks (range)[2]	13 (76) 37 (25, 62)
Complete TMA response, n (%) Median Duration of complete TMA response, weeks (range)[2]	11 (65) 38 (25, 56)
TMA event-free status, n (%)	15 (88)
Daily TMA intervention rate, median (range) Before eculizumab On eculizumab treatment	 0.88 (0.04, 1.59) 0 (0, 0.31)
Change in eGFR (mL/min/1.73 m^2) at 26 weeks, median (range)	+20 (-1, 98)
eGFR improvement ≥15 mL/min/1.73 m^2, n (%)[2] Median duration of eGFR improvement, days (range)[2]	9 (53) 251 (70-392)

1. Calculated at each post-dose day of measurement (excluding Days 1 to 4) through Week 26 using a repeated measurement ANOVA model.
2. At data cut-off (September 8, 2010).

Renal function, as measured by median eGFR, was improved during Soliris therapy. Four of the five patients who required dialysis at study entry were able to discontinue dialysis for the duration of Soliris treatment, and one patient developed a new dialysis requirement.

In aHUS Study 1, responses to Soliris were similar in patients with and without identified mutations in genes encoding complement regulatory factor proteins.

aHUS Sensitive to PE/PI (aHUS Study 2)

aHUS Study 2 enrolled patients undergoing chronic PE/PI who generally did not display hematologic signs of ongoing thrombotic microangiopathy (TMA). All patients had received PT at least once every two weeks, but no more than three times per week, for a minimum of eight weeks prior to the first Soliris dose. Patients on chronic dialysis were permitted to enroll in aHUS Study 2. The median patient age was 28 years (range: 13 to 63 years). Patients enrolled in aHUS Study 2 had an ADAMTS-13 level above 5%. Seventy percent of patients had an identified complement regulatory factor mutation or auto-antibody. Table 11 summarizes the key baseline clinical and disease-related characteristics of patients enrolled in aHUS Study 2.

Table 11: Baseline Characteristics of Patients Enrolled in aHUS Study 2

Parameter	aHUS Study 2 N=20
Time from aHUS diagnosis until screening in months, median (min, max)	48 (0.66, 286)
Time from current clinical TMA manifestation until screening in months, median (min, max)	9 (1, 45)
Baseline platelet count (× 10^9/L), median (range)	218 (105, 421)
Baseline LDH (U/L), median (range)	200 (151, 391)

Patients in aHUS Study 2 received Soliris for a minimum of 26 weeks. After completion of the initial 26-week treatment period, most patients continued to receive Soliris by enrolling into an extension study. In aHUS Study 2, the median duration of Soliris therapy was approximately 40 weeks (range: 26 to 52 weeks). Table 12 summarizes the efficacy results for aHUS Study 2.

Table 12: Efficacy Results for aHUS Study 2 in PE/PI-Sensitive aHUS

Efficacy Parameter	aHUS Study 2 N=20
TMA Event-free status n (%) (95% CI)[1]	16 (80) (56-94)
Daily TMA intervention rate, median (range) Before eculizumab On eculizumab treatment	 0.23 (0.05, 1.09) 0
Change in platelet count from baseline through week 26 (× 10^9/L), least square mean (95% CI)[1]	5 (-17.5 - 28)
Hematologic normalization[2], n (%) Median duration of hematologic normalization, weeks (range)[3]	18 (90) 38 (22, 52)
Complete TMA response, n (%) Median duration of complete TMA response, weeks (range)[3]	5 (25) 32 (12, 38)
Change in eGFR (mL/min/1.73 m^2) at 26 weeks, median (range)	+5 (-1, 20)
eGFR improvement ≥15 mL/min/1.73 m^2, n (%)	1 (5)

1. Calculated at each post-dose day of measurement (excluding Days 1 to 4) through Week 26 using a repeated measurement ANOVA model.
2. In aHUS Study 2, 85% of patients had normal platelet counts and 80% of patients had normal serum LDH levels at baseline, so hematologic normalization in this population reflects maintenance of normal parameters in the absence of PE/PI.
3. At data cut-off (October 10, 2010).

In aHUS Study 2, responses to Soliris were similar in patients with and without identified mutations in genes encoding complement regulatory factor proteins. Reduction in terminal complement activity was observed in all patients after commencement of Soliris. Soliris reduced signs of complement-mediated TMA activity, as shown by an increase in mean platelet counts from baseline to 26 weeks. Platelet counts were maintained at normal levels despite the elimination of PE/PI. The mean platelet count was 229 ± 78 x10^9/L at baseline, and 233 ± 69 x10^9/L at week 26. Renal function, as measured by median eGFR, was maintained during Soliris therapy. No patient required new dialysis with Soliris.

Retrospective Study in Patients with aHUS (aHUS Study 3)

The efficacy results for the aHUS retrospective study (aHUS Study 3) were generally consistent with results of the two prospective studies. Soliris reduced signs of complement-mediated TMA activity, as shown by an increase in mean platelet counts from baseline. Mean platelet count increased from 171 ± 83 x10^9/L at baseline to 233 ±109 x10^9/L after one week of therapy; this effect was maintained through 26 weeks (mean platelet count at week 26: 254 ± 79 x10^9/L).

A total of 19 pediatric patients (ages 2 months to 17 years) received Soliris in aHUS Study 3. The median duration of Soliris therapy was 16 weeks (range 4 to 70 weeks) for children < 2 years of age (n=5), 31 weeks (range 19 to 63 weeks) for children 2 to <12 years of age (n=10), and 38 weeks (range 1 to 69 weeks) for patients 12 to 17 years of age (n=4). Fifty three percent of pediatric patients had an identified complement regulatory factor mutation or auto-antibody.

Overall, the efficacy results for these pediatric patients appeared consistent with what was observed in patients enrolled in aHUS Studies 1 and 2 (Table 13). No pediatric patient required new dialysis during treatment with Soliris.

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Table 13: Efficacy Results in Pediatric Patients Enrolled in aHUS Study 3

Efficacy Parameter	<2 yrs (n=5)	2 to <12 yrs (n=10)	12 to <18 yrs (n=4)	Total (n=19)
Platelet count normalization, n (%)[1]	4 (80)	10 (100)	3 (75)	17 (89)
Hematologic Normalization, n (%)	2 (40)	5 (50)	1 (25)	8 (42)
Complete TMA response, n (%)	2 (40)	5 (50)	1 (25)	8 (42)
Daily TMA intervention rate, median (range)				
Before eculizumab	1 (0, 2)	<1 (0.07, 1.46)	<1 (0, 1)	0.31 (0.00, 2.38)
On eculizumab treatment	<1 (0, <1)	0 (0, <1)	0 (0, <1)	0.00 (0.00 , 0.08)
Patients with eGFR improvement ≥15 mL/min/1.73 m^2, n (%)[2]	2 (40)	6 (60)	1 (25)	9 (47)

1. Platelet count normalization was defined as a platelet count of at least 150,000 X 10^9/L on at least two consecutive measurements spanning a period of at least 4 weeks.
2. Of the 9 patients who experienced an eGFR improvement of at least 15 mL/min/1.73 m^2, one received dialysis throughout the study period and another received eculizumab as prophylaxis following renal allograft transplantation.

16 HOW SUPPLIED/STORAGE AND HANDLING

Soliris (eculizumab) is supplied as 300 mg single-use vials containing 30 mL of 10 mg/mL sterile, preservative-free Soliris solution per vial.

Soliris vials must be stored in the original carton until time of use under refrigerated conditions at 2-8° C (36-46° F) and protected from light. Do not use beyond the expiration date stamped on the carton. Refer to *Dosage and Administration (2)* for information on the stability and storage of diluted solutions of Soliris.

DO NOT FREEZE. DO NOT SHAKE.

NDC 25682-001-01 Single unit 300 mg carton: Contains one (1) 30 mL vial of Soliris (10 mg/mL).

17 PATIENT COUNSELING INFORMATION

See FDA-approved patient labeling (Medication Guide).

Prior to treatment, patients should fully understand the risks and benefits of Soliris, in particular the risk of meningococcal infection. Ensure that patients receive the Medication Guide.

Patients should be informed that they are required to receive a meningococcal vaccination at least 2 weeks prior to receiving the first dose of Soliris, if they have not previously been vaccinated. They are required to be revaccinated according to current medical guidelines for meningococcal vaccine use while on Soliris therapy. Patients should also be informed that vaccination may not prevent meningococcal infection. Patients should be educated about any of the signs and symptoms of meningococcal infection, and strongly advised to seek immediate medical attention if these signs or symptoms occur. These signs and symptoms are as follows:

- headache with nausea or vomiting
- headache and a fever
- headache with a stiff neck or stiff back
- fever of 103° F (39.4° C) or higher
- fever and a rash
- confusion
- muscle aches with flu-like symptoms
- eyes sensitive to light

Patients should be informed that they will be given a Soliris Patient Safety Information Card that they should carry with them at all times. This card describes symptoms which, if experienced, should prompt the patient to immediately seek medical evaluation.

Patients should also be informed that there may be an increased risk of other types of infections, particularly those due to encapsulated bacteria. Parents or caregivers of children receiving Soliris for the treatment of aHUS should be informed that their child should be vaccinated against *Streptococcus pneumoniae* and *Haemophilus influenza* type b (Hib) according to current medical guidelines.

Patients with PNH should be informed that they may develop hemolysis due to PNH when Soliris is discontinued and that they will be monitored by their healthcare professional for at least 8 weeks following Soliris discontinuation. Patients with aHUS should be informed that there is a potential for TMA complications due to aHUS when Soliris is discontinued and that they will be monitored by their healthcare professional for at least 12 weeks following Soliris discontinuation. Patients who discontinue Soliris should be instructed to keep the Soliris Patient Safety Information Card with them for three months after the last Soliris dose, because the increased risk of meningococcal infection persists for several weeks following discontinuation of Soliris.

Manufactured by:

Alexion Pharmaceuticals, Inc.
352 Knotter Drive
Cheshire, CT 06410 USA

US License Number 1743

This product, or its use, may be covered by one or more US patents, including U.S. Patent No. 6,355,245 in addition to others including patents pending.

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MEDICATION GUIDE

Soliris® (so-leer-is)
(eculizumab)

Read the Medication Guide before you start Soliris and before each infusion. This Medication Guide does not take the place of talking with your doctor about your medical condition or your treatment. Talk to your doctor if you have any questions about your treatment with Soliris.

What is the most important information I should know about Soliris?

Soliris is a medicine that affects your immune system. Soliris can lower the ability of your immune system to fight infections.

- **Soliris increases your risk of getting serious and life-threatening meningococcal infections.**

  Meningococcal infections may quickly become life-threatening and cause death if not recognized and treated early.

1. **You must receive a meningococcal vaccine at least 2 weeks before your first dose of Soliris unless you have already had this vaccine. If your doctor decides that urgent treatment with Soliris is needed, you should receive a meningococcal vaccine as soon as possible.**
2. **If you had a meningococcal vaccine in the past, you might need a booster dose before starting Soliris. Your doctor will decide if you need another dose of a meningococcal vaccine.**
3. **A meningococcal vaccine does not prevent all meningococcal infections. Call your doctor or get emergency medical care right away if you get any of these signs and symptoms of a meningococcal infection:**
   - **headache with nausea or vomiting**
   - **headache and a fever**
   - **headache with a stiff neck or stiff back**
   - **fever of 103° F (39.4° C) or higher**
   - **fever and a rash**
   - **confusion**
   - **muscle aches with flu-like symptoms**
   - **eyes sensitive to light**

Your doctor will give you a **Patient Safety Card** about the risk of meningococcal infection. Carry it with you at all times during treatment and for 3 months after your last Soliris dose. Your risk of meningococcal infection may continue for several weeks after your last dose of Soliris. It is important to show this card to any doctor or nurse who treats you. This will help them diagnose and treat you quickly.

Soliris is only available through a program called the Soliris REMS. Before you can receive Soliris, your doctor must:

- enroll in the Soliris REMS program
- counsel you about the risk of meningococcal infection
- give you information about the symptoms of meningococcal infection
- give you a **Patient Safety Card** about your risk of meningococcal infection, as discussed above.
- make sure that you are vaccinated with a meningococcal vaccine

Soliris may also increase the risk of other types of serious infections. If your child is treated with Soliris, make sure that your child receives vaccinations against *Streptococcus pneumoniae* and *Haemophilus influenza* type b (Hib).

What is Soliris?

Soliris is a prescription medicine called a monoclonal antibody. Soliris is used to treat people with:

- a disease called Paroxysmal Nocturnal Hemoglobinuria (PNH). PNH affects red blood cells.
- a disease called atypical Hemolytic Uremic Syndrome (aHUS). aHUS affects the blood system, kidney, and sometimes other body organs.

Soliris works by blocking part of your immune system. This can help your symptoms but it can also increase your chance for infection.

It is important that you:

- have all recommended vaccinations before you start Soliris
- stay up-to-date with all recommended vaccinations during treatment with Soliris

Who should not receive Soliris?

Do not receive Soliris if you:

- have a meningococcal infection
- have not been vaccinated against meningitis infection, unless your doctor decides that urgent treatment with Soliris is needed. See "What is the most important information I should know about Soliris?"

What should I tell my doctor before receiving Soliris?

Before receiving Soliris, tell your doctor if you:

- have an infection or fever
- are pregnant or plan to become pregnant. It is not known if Soliris will harm your unborn baby.
- are breastfeeding or plan to breastfeed. It is not known if Soliris passes into your breast milk.

Tell your doctor about all the medicines you take, including prescription and non-prescription medicines, vitamins, and herbal supplements.

Know the medicines you take. Keep a list of them to show your doctor and pharmacist when you get a new medicine.

How will I receive Soliris?

- Soliris is given through a vein (I.V. or intravenous infusion) usually over 35 minutes. If you have an allergic reaction during your Soliris infusion, your doctor may decide to give Soliris more slowly or stop your infusion.
- If you are an adult, you will usually receive a Soliris infusion by your doctor:
  - weekly for five weeks, then
  - every 2 weeks
- If you are less than 18 years of age, your doctor will decide how often you will receive Soliris depending on your age and body weight.
- After each infusion, you should be monitored for one hour for allergic reactions. See "What are the possible side effects of Soliris?"
- If you forget or miss a Soliris infusion, call your doctor right away.
- **If you have PNH, your doctor will need to monitor you closely for at least 8 weeks after stopping Soliris. Stopping treatment with Soliris may cause breakdown of your red blood cells due to PNH.**

  Symptoms or problems that can happen due to red blood cell breakdown include:
  - drop in the number of your red blood cells
  - drop in your platelet count
  - confusion
  - chest pain
  - kidney problems
  - blood clots
  - difficulty breathing
- **If you have aHUS, your doctor will need to monitor you closely during and for at least 12 weeks after stopping treatment for signs of worsening aHUS symptoms or problems related to abnormal clotting (thrombotic microangiopathy).**

  Symptoms or problems that can happen with abnormal clotting may include:
  - stroke
  - confusion
  - seizures
  - chest pain (angina)
  - difficulty breathing
  - kidney problems
  - swelling in arms or legs
  - a drop in your platelet count

What are the possible side effects of Soliris?

Soliris can cause serious side effects, including:

- **See "What is the most important information I should know about Soliris?"**

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- **Serious allergic reactions.** Serious allergic reactions can happen during your Soliris infusion. Tell your doctor or nurse right away if you get any of these symptoms during your Soliris infusion:
  - chest pain
  - trouble breathing or shortness of breath
  - swelling of your face, tongue, or throat
  - feel faint or pass out

  If you have an allergic reaction to Soliris, your doctor may need to infuse Soliris more slowly, or stop Soliris. See "How will I receive Soliris?"

Common side effects in people with PNH treated with Soliris include:

- headaches
- runny nose and colds
- sore throat
- back pain
- nausea

Common side effects in people with aHUS treated with Soliris include:

- high blood pressure
- common cold (upper respiratory infection)
- diarrhea
- headache
- nausea and vomiting
- low red blood cell count
- low white blood cell count
- urinary tract infection

Tell your doctor about any side effect that bothers you or that does not go away. These are not all the possible side effects of Soliris. For more information, ask your doctor or pharmacist.

Call your doctor for medical advice about side effects. You may report side effects to FDA at 1-800-FDA-1088.

General information about Soliris

Medicines are sometimes prescribed for conditions other than those listed in a Medication Guide. This Medication Guide summarizes the most important information about Soliris. If you would like more information, talk with your doctor. You can ask your doctor or pharmacist for information about Soliris that is written for healthcare professionals.

What are the ingredients in Soliris?

Active ingredient: eculizumab

Inactive ingredients: sodium phosphate monobasic, sodium phosphate dibasic, sodium chloride, polysorbate 80 (vegetable origin) and Water for Injection.

This Medication Guide has been approved by the U.S. Food and Drug Administration.

Manufactured by Alexion Pharmaceuticals, Inc. 352 Knotter Drive, Cheshire, CT 06410 USA.

Revised: 09/2011

100172v4.0
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Europäisches Patentamt
European Patent Office
Office européen des brevets

Anmeldenummer	Application No.	Numéro de la demande:
	05712607.0	

INFORMATION

Die mündliche Verhandlung am:	The oral proceedings of:	La procédure orale du:
	15.05.14	
hat ergeben:	resulted in:	fut conclue comme suit:
☐ Das europäische Patent wird widerrufen, da wenigstens ein Einspruchsgrund der Aufrechterhaltung des europäischen Patents entgegensteht (Art. 101(2) EPÜ).	The European patent is revoked because at least one ground for opposition prejudices the maintenance of the European patent (Art. 101(2) EPC)	Le brevet européen est révoqué car au moins un motif d'opposition s'oppose au maintien du brevet européen (art. 101(2) CBE).
☒ Das europäische Patent wird widerrufen, da unter Berücksichtigung der vom Patentinhaber im Einspruchsverfahren vorgenommenen Änderungen das europäische Patent und die Erfindung, die es zum Gegenstand hat, den Erfordernissen des EPÜ nicht genügen (Art. 101 (3) b) EPÜ).	The European patent is revoked because, account being taken of the amendments made by the patent proprietor during opposition proceedings, the patent and the invention to which it relates were found not to meet the requirements of the EPC (Art. 101(3)(b) EPC).	Le brevet européen est révoqué car il a été établi que, compte tenu des modifications apportées par le titulaire du brevet au cours de la procédure d'opposition, le brevet et l'invention qui en fait l'objet ne satisfont pas aux exigences de la Convention sur le brevet européen (art. 101(3)(b) CBE).
☐ Der Einspruch wird/Die Einsprüche werden zurückgewiesen (Art. 101(2) EPÜ).	The opposition(s) is/are rejected (Art. 101(2) EPC).	L'opposition est/Les oppositions sont rejetée(s) (art. 101(2) CBE).
☐ Es wird festgestellt, dass unter Berücksichtigung der vom Patentinhaber im Einspruchsverfahren vorgenommenen Änderungen das Patent und die Erfindung, die es zum Gegenstand hat, den Erfordernissen des Europäischen Patentübereinkommens genügen (Art. 101(3)(a) EPÜ).	Account being taken of the amendments made by the patent proprietor during the opposition proceedings, the patent and the invention to which it relates are found to meet the requirements of European Patent Convention (Art. 101(3)(a) EPC).	Il est établi que, compte tenu des modifications apportées par le titulaire du brevet au cours de la procédure d'opposition, le brevet et l'invention qui en fait l'objet satisfont aux exigences de la Convention sur le brevet européen (art. 101(3)(a) CBE).
☐ Die während der mündlichen Verhandlung eingereichten Änderungen sind beigefügt.	Amendments filed during oral proceedings are annexed.	Les modifications soumises pendant la procédure orale sont annexées.

15.05.2014
Datum / Date / Date

Unterschrift / Signature / Signature

NB: Dieses Formblatt ist nur als Information zu sehen. Die schriftliche Entscheidung hat Vorrang.
This form is provided for the sake of information only. The written decision prevails.
Le présent formulaire n'a qu'une valeur informative. La décision écrite prévaut.

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Application No.: 05 712 607.0

Patent No.: EP-B-1 720 571

Direct Decision:

☐ yes ☒ no

Revocation of the European Patent (Art. 101(3)(b) EPC)

The Opposition Division - at the oral proceedings dated 15.05.2014 - has decided:

European Patent No. EP-B- 1 720 571 is revoked.

The Grounds for the decision (Form 2916) are enclosed.

24. 06. 14
Date

Chairman	1st Examiner	2nd Examiner	Legally qualified member
Cilensek, Zoran	Chapman, Rob	Weikl, Martina	

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Vossius & Partner
Siebertstrasse 4
81675 München
ALLEMAGNE

Date
01-07-2014

Reference	Application No./Patent No.
M2367 EP	05712607.0 - 1412 / 1720571
Applicant/Proprietor	
ALEXION PHARMACEUTICALS, INC.	

Decision revoking the European patent (Art. 101(2), (3)(b) EPC)

The Opposition Division - at the oral proceedings dated 15.05.14 - has decided:

The European Patent No. 1720571 is revoked.

☐ Additional decision:
The opposition of the opponent(s) ____ is rejected as inadmissible.
Apportionment of costs (Art. 104(1) EPC) as indicated in the grounds for the decision.

The reasons for the decision are enclosed.

Appeal
This decision is open to appeal. Attention is drawn to the attached text of Articles 106 to 108 EPC and Rules 97 and 98 EPC.

Opposition Division:

1st Examiner:	2nd Examiner:	Chairman:
Chapman R	Weikl M	Cilensek Z

Enclosures: Reasons for the decision (Form 2916, 10 pages)
Text of Articles 106 - 108 and Rules 97 - 98 EPC (EPO Form 2019)
☒ Minutes of oral proceedings

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EPO Form 2331 12.07 (26/06/14) REVO

to EPO postal service: 26.06.14
CC20871

1 Facts and Submissions

Reference is made to the following documents cited by the Opponent; the numbering will be adhered to for the rest of the proceedings.

D1 Hillmen et al., (2004) The New England Journal of Medecine, 350:552-9

D2 Jane Salodof MacNeil (2002) Medscape Medical News

D3 Hillmen et al., (2003) British Journal of Hematology 121 (Suppl. 1) 87-95, Abstract 257

D4 Hillmen et al., (2002) Blood. 100(11), Abstract 154

D5 Hillmen et al., (2003) Journal of The American Society of Hematology 102(11) Abstract 1858

D6 Rother et al., (2003) Molecular Immunology. 40, p 197, Abstract 54

D7 Rosse et al., (2004) American Society of Hematology, p 48-62

D8 Biotechnology News (2004) 24(30), p 10

D9 US 6,355,245

D10 Thomas et al., (1996) Molecular Immunology, Vol. 33, No. 17/18, p 1389-1401

D11 Drugs of the Future (2004) 29(7): 673-676

D12 Hillmen et al., British Journal of Haematology, 125 (Suppl. 1), 1-72, Abstract 5

D13 Kaplan, (2002) Current Opinion in Inv. Drugs, vol. 3, no. 7, pages 1017-1023

D14 Declaration of Leonard Bell (2014)

D15 Packaging insert/Datasheet from Eculizumab (Alexion, 2011)

1.1 European patent EP1720571 (hereinafter designated the patent) entitled "Method of treating hemolytic disease", was published in the official bulletin on 13.06.2012 and a corrigendum was issued on 24.10.2012. The Proprietor (hereafter designated 'P') is Alexion Pharmaceuticals, Inc., Cheshire, US.

Said patent is based on European application 05712607.0, filed on 03.02.2005 and claims the priority from US 10/771,552 filed on 03.02.2004. The International publication number is WO2005/074607.

1.2 Notice of opposition against the above-mentioned patent was received from the following party (hereinafter named the Opponent or 'O').

1. Pfizer Inc., 235 East 42nd Street, New York 10017, US (filed online with letter dated 12.03.2013, received on 12.03.2013).

1.3 The O requested the revocation of the patent as granted in its entirety on the basis of Articles 100(a)(b)(c) because the subject-matter granted was not novel, inventive, or disclosed in a manner sufficiently clear and complete to be carried out by a skilled person (Articles 52(1),54,56 and 83 EPC), and comprised subject-matter that extended beyond the application as originally filed (Article 123(2) EPC). The O filed D1-D12 therewith.

1.4 In their letter dated 07.10.2013, the O withdrew their request of opposition against the above-mentioned patent.

1.5 With their letter dated 09.10.2013, the P requested that their patent be maintained as granted and that the opposition procedure be closed. The P requested oral proceedings in case their respective request would not be granted.

1.6 The Opposition Division (OD) issued a non-binding preliminary opinion with their official communication of 14.01.2014, based on the grounds of opposition raised by the O, and which the OD considered pertinent to pursue of its own motion, in accordance with Article 114 EPC. Oral proceedings were appointed for 15.05.2014.

1.7 The P submitted document D13 and arguments in support of maintenance of the patent as granted with their letter of 30.12.2013.

1.8 The OD reissued their non-binding preliminary opinion in order to account for the P's arguments that had not been presented to the OD before issuance of the summons to oral proceedings. The date scheduled for oral proceedings was maintained.

1.9 In their letter of 17.013.2014, the P submitted further arguments, a new main request (MR) and an auxiliary request (AR1) to replace the claims of the opposed patent. The P requested maintenance of the scheduled oral proceedings, in case the OD still not consider that any of the new requests overcome the objections raised.

1.10 Oral proceedings took place on 15.05.2014 in the presence of the P alone, since the O had withdrawn from proceedings. At the proceedings the Chairman (C) informed the P that the examination of the opposition was thus being continued of the OD's own motion. The P maintained their requests on file and submitted a declaration (D14), and a datasheet (D15) to support the subject-matter claimed.

1.11 At the end of oral proceedings, the OD announced the decision to revoke the patent in full in the absence of further allowable requests, because AR1 did not fulfill the requirements of Article 56 EPC.

2 Reasons for the Decision

Admissibility

2.1 The opposition complied with Articles 99 and 100 EPC. No objections were raised. The opposition is allowable.

Procedural matters

2.2 The O formally withdrew their opposition to the patent on 07.10.2013.

2.3 The OD considered that the arguments and documents submitted by the O to be valid grounds for opposition and thus decided to pursue opposition *proceedings of its own motion* (Article 114(1) EPC; GL D, VII-5.2;5.3).

2.4 The P filed D14, a declaration of a technical expert, and D15, a datasheet, during oral proceedings. The OD decided to admit D14 and D15 in to the proceedings, since they provided background information to the subject-matter claimed.

The decision is being carried out on the **following requests:**

Main Request

Description, Pages

3-13	of the patent specification

Claims, Numbers

1-8	received on	18-03-2014	with letter of	18-03-2014

Drawings, Sheets

1/11-11/11	of the patent specification

Auxiliary Request 1

Description, Pages

3-13 of the patent specification

Claims, Numbers

1-8 received on 18-03-2014 with letter of 18-03-2014

Drawings, Sheets

1/11-11/11 of the patent specification

Main Request (MR)

The MR differs from the patent specification through the modification of the route of administration to explicitly state 'intravenous infusion', thus replacing the term 'intravenously' in claim 1 of the granted patent.

Rule 80, Articles 84, 123(2) and 123(3) EPC

2.5 The OD decided that the MR complies with Rule 80, Articles 84, 123(2) and 123(3) EPC since the term 'intravenous infusion' is clearly stated as the route of administration in the example on which it is based (p.32, li.13) in the application. The OD found that it would be obvious that all other steps in the claim comprising the same substance would be administered via the same route unless otherwise stated in the example. All objections raised were thus overcome.

Lack of novelty (Articles 100(a) and 54 EPC)

2.6 Objections were raised on grounds of lack of novelty of claims 1 and 5 with regard to D1.

2.7 D1 published on 05.02.2004 discloses all the features of claims 1-8 (see Methods and section 'Biochemical indicators of hemolysis' on p.552-556 and Fig.3A) but is published after the date of priority of the patent.

2.8 The patent claims priority from US10/771552, filed on 03.02.2004, and appears to disclose identical subject-matter to the application filed on 03.02.2005 on which the patent is based.

2.9 The OD considered that since the priority document discloses the claimed dosage regimen for treatment of complement breakthrough via intravenous infusion, D1 is not considered prior art and the subject-matter of claims 1 and 5 is new.

Lack of inventive step (Articles 56 and 100(a) EPC)

2.10 The OD objected to the MR on grounds of a lack of inventive step, in light of D5 alone as the closest prior art to the subject matter of claims 1 and 5, or in combination with any of D3, D4 or D6.

2.11 The P concurred that D5 should be considered the closest prior art, as it is directed to the same technical purpose, i.e. it is directed to treating a patient afflicted with PNH suffering a breakthrough in hemolytic activity.

2.12 Document D5 discloses the continuation of a pilot study comprising:

1. the use of eculizumab
2. the treatment of a patient affected with PNH
3. intravenous infusion
4. administration of 900mg bi-weekly
5. reducing the dosing interval slightly in response to breakthrough and thus resolving the symptoms of PNH.

2.13 The P stated that the difference between claim 1 and document D5 can therefore be considered to be that D5 does not disclose a complete dosage schedule. D5 is directed only to the maintenance stage of treatment, i.e. stages (iii) and (iv) of claim 1, and does not teach the essential induction phase stages (i) and (ii).

2.14 The technical effect associated with this difference is a complete dosage schedule for treating PNH and modifications for the alleviation of symptoms associated with a breakthrough in hemolytic activity.

2.15 The problem to be solved can therefore be considered to be the provision of a complete dosage regimen for maintaining patients with PNH symptom free.

2.16 The solution provided in the granted claims is not obvious, since the essential pre-loading stage is not taught in D5.

2.17 The P referred to the declaration D14, §4, wherein it is stated that the administration of 900 mg eculizumab at week five is essential for achieving an adequate baseline of complement inhibition that can be maintained with bi-

weekly 900 mg eculizumab doses thereafter. The P referred to the dosage regimen and provisions for its use, stated in the packaging datasheet D15 that accompanies eculizumab, as proof that this is the regimen used in practice.

The P thus concluded that D5 alone does not teach the features necessary to provide a complete dosage regimen for maintaining patients with PNH symptom free.

2.18 Furthermore, the P stated that D5 combined with any of D3,D4 or D6 would not permit the skilled person to arrive at the regimen claimed:

Documents D3 and D4 disclose a pilot study comprising the following features:

1. the use of eculizumab
2. the treatment of a patient affected with PNH
3. intravenous infusion
4. administration of 600mg each week for 4 weeks
5. administration of 900mg bi-weekly thereafter

The skilled person combining the teaching of D5 with any of D3 or D4 would not arrive at the dosage regimen claimed, since the administration of 900 mg eculizumab at week 5 is not taught.

2.19 The P explained that the skilled person would not arrive at the claimed dosage regimen when combining the teaching of D5 with D6:

Document D6 discloses the following features:

1. the use of eculizumab
2. the treatment of a patient affected with PNH
3. intravenous infusion
4. administration of 600mg each week for 4 weeks
5. administration of 900mg a week later
6. administration of 900mg bi-weekly thereafter

2.20 D6 teaches the administration of 900 mg eculizumab at week 5. However, the skilled person following the dosage schedule of D6 and experiencing breakthrough of hemolytic activity in PNH patients would not reduce the dosage interval to 900 mg eculizumab every 12 days: D5 merely teaches the 'slight' reduction of the dose in this situation. The P claimed that a reduction in dosage interval from 14 to 12 days cannot be considered slight, as it amounts

to around 15%. Moreover, the skilled person with a knowledge of D13 (p. 1021, col.1, §1 - 4) would know that the behaviour of the complement system is difficult to predict, and would not reduce the intervals by so much, and certainly not to 12 days, since shortening of dosing intervals may not result in an effective treatment by eculizumab.

2.21 The OD argued that the replacement of one regimen with another is a matter of routine experimentation, as supported by case law of the Technical Board of Appeal T1979/09, wherein under point 7.3.1 it is stated *'It is well known that in designing dosage regimen the two major parameters that can be adjusted are the dose itself, i.e. the quantity of drug administered, and the dosing frequency, i.e. the time interval between doses.'*

and further under point 7.3.2, *'In the present case the amount of active ingredient remains the same as the amount used in D5 and there is no evidence on file of any unexpected technical effect caused by administering the red vine leave extract once daily. The board considers that it is within the competence of the skilled person to find out the optimal dosage regime. Consequently, the replacement of a dosage regime by another dosage regime for the same purpose is considered to be a matter of routine experimentation and cannot be seen as involving an inventive step.'*

2.22 The P disagreed with this interpretation, since the present regimen comprises 4 different steps as stated in the claim, and thus cannot be compared to the simple regimen considered in T1979/09.

2.23 The OD argued that D13 is not directed to the same technical problem posed. Claim 1 is clearly directed to patients suffering breakthrough who are already receiving a standard regimen, as disclosed in D6. D5 clearly discloses how cases in which breakthrough is experienced can be resolved. D5, alike D6 was published after D13 and represents the actual stand of knowledge at the time of priority filing. The OD therefore considers that given the nature of D13 in reporting preliminary research results, and the subsequent publications D5 and D6, the skilled person would not consider D13 when selecting a dosage regimen for the indication presently claimed.

2.24 The P argued that neither D5 alone or combined with D6 any other cited document would anticipate the subject-matter of claim 1, however, if the skilled person were to combine D5 with other documents, he would select D3 or D4 as these relate to less complicated teachings.

2.25 The OD considers D6 to be the most recent and detailed teaching of the cited prior art D3,D4 and D6. The skilled person would therefore not consider D6 too complicated, rather to be the most up-to-date teaching for the treatment of PNH based on the pilot studies described in D2,D3 and D4.

2.26 The OD finds that according to D5, transient breakthroughs in complement activity associated with increased LDL, hemoglobinuria and mild symptoms in an extension trial of patients receiving the dosage regimen described in D6, were known to be preventable by a slight decrease in the dosing interval. The skilled person would have chosen and combined the teachings of D5 and D6 because they both describe the treatment of PNH with eculizumab. D6 discloses the latest details of a pilot study comprising the dose loading stages of parts (i) - (iii) of claim 1, whereas D5 describes an extension to what appears to be the same pilot study, wherein patients experiencing breakthrough of hemolytic activity are treated by a slight reduction in dosing interval, which successfully resolves their symptoms.

2.27 The OD thus concludes that the skilled person would be taught by D5 to reduce the dosage interval to solve the problem of breakthrough in PNH patients receiving the priming dosage regimen described in D6. The skilled person would reduce the interval 'slightly', i.e. by at say least 10%, to 12 days to resolve the symptoms, as suggested by D5.

The subject-matter of the MR therefore does not meet the requirements of Article 56 EPC.

Auxiliary Request 1 (AR1)

AR1 differs from the MR in that a result to be achieved by the dosage regimen of claim 1 of the MR has been added, i.e. a reduction of serum hemolytic activity (cell lysis) below 20%.

Rule 80, Articles 84, 123(2) and 123(3) EPC

2.28 The OD decided that AR1 complies with Rule 80, Articles 84, 123(2) and 123 (3) EPC since the term 'intravenous infusion' is clearly stated as the route of administration in the example on which it is based (p.32, li.13) in the application. The OD found that it would be obvious that all other steps in the claim comprising the same substance would be administered via the same route unless otherwise stated in the example. The feature that cell lysis is reduced by at least 20% was deemed to find a basis on p.38, li.11-17.

Lack of inventive step (Articles 56 and 100(a) EPC)

2.29 The OD objected to AR1 on grounds of a lack of inventive step, in light of D5 as the closest prior art to the subject matter of claims 1 and 5, in combination with D6.

2.30 The P argued that neither D5 nor D6 refer to any threshold value to be used to assess breakthrough of hemolytic activity.

2.31 The problem to be solved may thus be considered to be the provision of a dosage regimen that permits the maintenance of complement activity below a threshold that prevents breakthrough.

2.32 The solution is the dosage regimen of AR1. Neither D5 or D6 teach how to assess breakthrough by the parameters claimed. The application clearly discloses how serum hemolytic activity can be used to identify patients experiencing breakthrough, and that reducing this activity below 20% correlates resolves the symptoms of PNH (p.38 and Fig.8).

2.33 The OD noted that Fig.8 shows at least 4 patients with hemolytic activity above 20%, yet only 2 are described as experiencing breakthrough. The claimed parameter would thus appear to be a non-technical feature of the claim whose relevance is unclear, since patients with serum hemolytic activity above this threshold do not necessarily appear to demonstrate breakthrough.

2.34 The P explained that classic symptoms such as urine colour (hemoglobinuria) were signs of hemolytic breakthrough. In D5, LDH, hemoglobinuria and other mild symptoms were measured, but these are not direct methods of measuring serum complement activity. The parameter claimed thus identifies patients that are successfully treated.

2.35 The OD finds that the technical effect of the dosage regimen claimed is the treatment of PNH. The claim is directed to a dosage regimen and not to a method of diagnosis.

Fig.8 of the application demonstrates that patients with 0 - 30% serum hemolytic activity do not exhibit symptoms of PNH in the group tested. Not all patients are maintained below 20% activity, suggesting that the regimen claimed may not even achieve the technical effect claimed.

Furthermore, alternative methods for monitoring complement breakthrough are disclosed in Fig.4 of the application, which are the same as those used in D5.

The parameter of reducing hemolytic activity below 20% does not appear to provide a further technical feature associated with any surprising technical effect, but may at best be another read-out to confirm the same resolution of breakthrough already recorded in D5.

2.36 The OD therefore concludes that AR1 does not meet the requirements of Article 56 EPC.

Decision

3 Taking into account the amendments made by the P, the patent according to the Main Request or Auxiliary Request 1 was found not to comply with the requirements of Article 56 EPC. The OD decided to revoke the amended patent in accordance with the provisions of Article 101(3)(b) EPC.

Tracking Information

EP/ECT/PCT No.:	**057126070**
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Adress:	Siebertstrasse 4
City:	81675 München
Country:	DE
Formtype:	2331/2019/2916
Send Date:	Tuesday, July 01, 2014

Delivery Information:

Tracking Number:	1Z01F1210459362963
Status:	DELIVERED
Pickup Date:	Tuesday, July 01, 2014
Delivery Date:	Wednesday, July 02, 2014
Deposited at:	OFFICE
Signed by (Name):	LEAXNER
Signature:	

Europäisches Patentamt
European Patent Office
Office européen des brevets

European Patent Office
80298 MUNICH
GERMANY

EPO - Munich
82

03. Juli 2014

Questions about this communication ?
Contact Customer Services at www.epo.org/contact

Formalities officer: Mr Consalvo Cavestri

Please specify the name of the formalities officer when contacting Customer Services by contact form or phone.

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ALLEMAGNE

Date
01-07-2014

Reference	Application No./Patent No.
M2367 EP	05712607.0 - 1412 / 1720571
Applicant/Proprietor	
ALEXION PHARMACEUTICALS, INC.	

EPA/EPO/OEB Formblatt/Form/Formulaire : 2936

Empfangsbescheinigung über den Zugang des vorstehend bezeichneten Schriftstücks
Acknowledgement of receipt of the document specified above
Récépissé du document spécifié ci-dessus

Unter Bezugnahme auf die Mitteilung im ABl EPA 7/2010, 377 wird gebeten, die Empfangsbescheinigung mit Empfangsdatum und Unterschrift zu versehen und **umgehend** an das EPA zurückzusenden:

With reference to the Notice in OJ EPO 7/2010, 377, you are requested to date and sign the acknowledgement of receipt and return it to the EPO **immediately**:

Conformément au communiqué paru au JO OEB 7/2010, 377, vous êtes prié d'indiquer sur le récépissé la date de réception du document, de signer le récépissé et de le renvoyer **sans délai** à l' OEB:

- **über die Online-Dienste des EPA** (als Anlage zu EPA Form 1038) / **through EPO Online Services** (as annex to EPO Form 1038) / **par les services en ligne de l'OEB** (en tant que pièce jointe au formulaire OEB 1038),
- **per Fax / by fax / par téléfax (+49 (0) 89 2399-4465 or +31 (0) 70 340-3016)**
- oder per Post / or by post / ou par courrier.

Empfangen am / Received on / Reçu le :

02. Juli 2014

Unterschrift / Signature /

RAINER VIKTOR
EUROPEAN PATENT ATTORNEY

Empfangsberechtigter/authorised recipient/
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DEUTSCHLAND

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CC20871

VOSSIUS & PARTNER
Patentanwälte Rechtsanwälte

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

EPO - Munich
75

08. Sep. 2014

European Patent Office

80298 MUNICH

EP 05 71 2607.0 / 1720571
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Our Ref.: M2367 OPP(EP)

München, September 8, 2014
WA/FLG/BUS

On behalf of Alexion Pharmaceuticals, Inc., 352 Knotter Drive, Cheshire, CT 06410, U.S.A.

and in accordance with Art. 106(1) EPC

Zur Kasse

1860,00 €

APPEAL

is lodged against the decision to revoke the European Patent No. 1720571.

Enclosed please find EPO Form 1010 covering the official fee for appeal.

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It is requested that the Decision of July 1, 2014 be reversed and the patent be maintained.

As an auxiliary measure only, it is requested that oral proceedings according to Art. 116 EPC be held.

Dr. Joachim Wachenfeld
European Patent Attorney

Enclosure:
EPO Form 1010

Samsung Bioepis Exhibit No. 1027
Page 382

Payment of fees and expenses

EPO - Munich 75

08. Sep. 2014

European Patent Office
Treasury and Accounts
80298 München
Germany

Please complete in typescript only

Alexion Pharmaceuticals, Inc.

Name of Payer

01 Vossius & Partner
(No. 31)

Payer's reference

M2367 OPP(EP)

Mode of payment

☐ Bank/Giro transfer [1]

Bank/Giro Office

Address

Siebertstraße 4
81675 München

02

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

Deposit account No.

2800.0321

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

03 EP 05 71 2607.0 - 1412

PCT

03

	Code		Currency	Amount
04	001	Filing fee – EP direct	EUR	
05	002	Search fee	EUR	
06	005	Designation fee(s)	EUR	
07	015	Claims fee(s) (Rules 45(1), 162(1) EPC)	EUR	
08	055	Additional copy	EUR	
09	006	Examination fee	EUR	
10	007	Fee for grant	EUR	
11	008	Additional fee for printing specification (more than 35 pages)	EUR	
12	033	Renewal fee for the 3rd year	EUR	
13	034	Renewal fee for the 4th year	EUR	
14	035	Renewal fee for the 5th year	EUR	
15	020	Filing fee – entry EP phase:	EUR	
16		Extension fee(s) For [4]:	EUR	
17	011	Appeal fee	EUR	1.860,00
18			EUR	
19			EUR	
20			EUR	
21			EUR	
22		Total	EUR	1.860,00

Signature
Dr. Joachim Wachenfeld
European Patent Attorney

Place, Date Munich, September 8, 2014, WA/FLG/BUS

EPO Form 1010 04.09

Europäisches Patentamt
European Patent Office
Office européen des brevets

European patent application No. 05712607.0

APPEAL for immediate attention

I. Findings

1. ☒ Appeal in which **more than one party** is involved (opposition procedure, except when all oppositions have been withdrawn). **Refer the case to the Board of Appeal without delay using EPO Form 2703.**

2. ☐ Appeal in which **only one party** is involved (examination procedure and opposition procedure if all oppositions have been withdrawn).

2.1 Requirements of Article 108 EPC

The **notice of appeal** has been filed within the time limit.	yes ☐	no ☐
The **appeal fee** has been paid within the time limit.	yes ☐	no ☐
The **statement of grounds** has been filed within the time limit.	yes ☐	no ☐

If **one** of the answers set out above is 'no' and if no request for re-establishment of rights under Article 122 EPC has been filed (see I.2.2), **refer the case to the Board of Appeal without delay using EPO Form 2703.** The Board of Appeal decides on the admissibility of the appeal.

If **all** the answers set out above are "yes", continue under II/III.2.

2.2 ☐ A request for re-establishment of rights into the appeal period has been filed. Refer the case to the SCAPE of the SIS Unit without delay.

2.3 Special cases which have to be referred to the Board of Appeal without delay using EPO Form 2703

- ☐ Withdrawal of appeal
- ☐ Request for correction of appeal (R. 139 EPC)
- ☐ Expiry of three-month time limit for interlocutory revision (Art. 109(2) EPC)

15-09-2014	Germano, Almut	
Date	Formalities Officer	Director/SCAPE (for information)

II. Appeal against the decision of the Formalities Officer

1. ☐ Re-establishment of rights (Guidelines E-VII, 2.2.7)
   - ☐ The conditions for granting re-establishment of rights are fulfilled. The request can be granted if this can be done within the three-month time limit of Article 109(2) EPC and if the conditions for granting interlocutory revision are fulfilled. Continue under II.2.
   - ☐ The request for re-establishment of rights cannot be granted. **Refer the case to the Board of Appeal without delay using EPO Form 2703.**

2. ☐ The appeal is allowable and well-founded. The decision under appeal is rectified (Art. 109(1) EPC). EPO Form 2710 is to be dispatched.
   - ☐ The decision under appeal is not rectified. **Refer the case to the Board of Appeal without delay using EPO Form 2703 (Art. 109(2) EPC).**

AG21431

3. Reimbursement of appeal fee

☐ No request for reimbursement of the appeal fee has been filed.
  ☐ The Formalities Officer orders reimbursement of the appeal fee of his own motion (R. 103 EPC, Guidelines E-X, 7.3).

☐ A request for reimbursement of the appeal fee has been filed.
  ☐ Reimbursement of the appeal fee is ordered (R. 103 EPC).
  ☐ The request for reimbursement of the appeal fee cannot be allowed. **Refer the case to the Board of Appeal without delay using EPO Form 2703 (R. 103(2) EPC).**

.....	.....	.....
Date	Formalities Officer	SCAPE (for information)

III. Appeal against the decision of the Examining/Opposition Division

1. ☐ Re-establishment of rights (Guidelines E-VII, 2.2.7)
  ☐ The conditions for granting re-establishment of rights are fulfilled. The request can be granted if this can be done within the three-month time limit of Article 109(2) EPC and if the conditions for granting interlocutory revision are fulfilled. Continue under III.2.
  ☐ The request for re-establishment of rights cannot be granted. **Refer the case to the Board of Appeal without delay using EPO Form 2703.**

2. ☐ **To the Examining Division** ☐ **To the Opposition Division**

.....	.....
Date	Formalities Officer

To the Formalities Officer

3. ☐ The appeal is allowable and well-founded. The decision under appeal is rectified (Art. 109(1) EPC). EPO Form 2710 is to be dispatched.
  ☐ The decision under appeal is not rectified. **Refer the case to the Board of Appeal without delay using EPO Form 2703 (Art. 109(2) EPC).**

4. Reimbursement of appeal fee

☐ No request for reimbursement of the appeal fee has been filed.
  ☐ The Division orders reimbursement of the appeal fee of its own motion (R. 103 EPC, Guidelines E-X, 7.3).

☐ A request for reimbursement of the appeal fee has been filed.
  ☐ Reimbursement of the appeal fee is ordered (R. 103 EPC).
  ☐ The request for reimbursement of the appeal fee cannot be allowed. **Refer the case to the Board of Appeal without delay using EPO Form 2703 (R. 103(2) EPC).**

.....	☐ Examining Division	☐ Opposition Division
Date		

.....	.....	.....	.....
Chairman	2nd Examiner	Primary Examiner	Legal Member

Samsung Bioepis Exhibit No. 1027
Page 385

Europäisches Patentamt
European Patent Office
Office européen des brevets

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ALLEMAGNE

Datum/Date
24.09.14

Zeichen/Reference/Référence	Anmeldung Nr./Application No./Demande n°//Patent Nr./Patent No./Brevet n°
M2367 EP APPR	05712607 / 1720571
Anmelder/Applicant/Demandeur//Patentinhaber/Proprietor/Titulaire	
ALEXION PHARMACEUTICALS, INC.	

File number: **T1897/14-3.3.04**

Commencement of proceedings before the Board of Appeal

The letter dated 08.09.14 filed by the patent proprietor
against the decision of the European Patent Office of 01.07.14
has been referred to Board of Appeal 3304.

The reference number is mentioned above.

Any further communications should be addressed to Directorate-General 3 of the European Patent Office and should quote this reference number.

The Registrar P. Cremona
Tel.: 089 / 2399 - 3341

Annex: procedural guidance
Registered letter

European Patent Office - Board of Appeal 3.3.04

These informal notes are provided by Board 3.3.04 for the guidance of the parties. They must be read in conjunction with and subject to the EPC and the Rules of Procedure of the Boards of Appeal (RPBA). Parties are expected to comply with them.

1. Board 3.3.04 endeavours to expedite the appeal procedure so far as possible. Parties are asked to assist the board in achieving this. To that end parties should observe the provisions of Articles 12, 13, 15 und 16 RPBA.

In particular parties should comply with the specific requirement in Article 12 (2) RPBA that the statement of grounds of appeal - and in *inter partes* cases the reply - should contain a party's complete case, specify expressly all the facts, arguments and evidence relied on, and be clearly and concisely presented.

Requests, both for sets of claims and for procedural measures, should also be filed with the statement of grounds of appeal or - as the case may be - the reply.

2. Extensions of time limits will in accordance with Article 12(5) RPBA only be exceptionally allowed following a written request explaining the exceptional circumstances and made sufficiently in advance.

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The four month time limit provided in Article 12(1)(b) RPBA for filing the respondent's reply to the statement of grounds of appeal will usually not be extended for reasons of fairness to the appellant who also has a four month non-extendable time limit for filing the statement of grounds of appeal.

3. Communications will be sent to the parties if the board considers it appropriate to do so. Such communications may deal with some but not necessarily all procedural and/or substantive matters. Any opinions of the board shall be regarded as provisional and non-binding for the board.

4. The date set for oral proceedings reflects the requirement to give at least two months notice, the availability of the three members assigned to the case and the availability of a suitable hearing room (the board has no permanently allocated room).

Therefore, the date will in the ordinary course only be changed if the board is satisfied that the reasons advanced are so serious as to justify the change (see "Notice of the Vice-President of Directorate-General 3 of the European Patent Office dated 16 July 2007 concerning oral proceedings before the boards of appeal of the EPO"). According to point 2.3 of the "Notice" the parties are moreover required to state why another representative cannot substitute for the representative prevented from attending the oral proceedings.

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Page 388

Oral proceedings will normally be appointed for a maximum length of one working day. It is the parties' responsibility to comply with the given time frame.

5. Despite the board's efforts to process pending appeals efficiently, the high number of pending cases has the consequence that the board is at present mainly dealing with cases filed during the years 2009 and 2010. Parties should currently allow for a delay of at least three years between filing and disposal of their appeals (unless acceleration was requested and allowed, see "Notice from the Vice-President Directorate-General 3 dated 17 March 2008 concerning accelerated processing before the Boards of Appeal").

Gabriele Alt
Chairwoman, Board 3.3.04

Samsung Bioepis Exhibit No. 1027
Page 389

VOSSIUS & PARTNER
Patentanwälte Rechtsanwälte

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

EPO - Munich
58

11. Nov. 2014

European Patent Office

80298 M U N I C H

T1897/14-3.3.04
(05 71 2607.0 / 1720571)
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Our Ref.: M2367 OPP(EP)

München, November 11, 2014
WA/FLG

Further to the appeal filed on September 8, 2014, against the decision of the Opposition Division to revoke the above-referenced European patent entitled "Method for treating haemolytic disease" issued on July 1, 2014, we now provide the grounds of appeal in accordance with Article 108 EPC.

It is requested that the patent is maintained for all designated contracting states based on the enclosed Main Request. Its subject-matter is patentable within the terms of the EPC. If the Board of Appeal does not see itself in a position to comply with our main request on the sole basis of our written submissions, we request consideration of auxiliary request 1.

The main request and auxiliary request 1 are identical to the main request and auxiliary request 1 dealt with in the impugned decision.

Should the Board be minded not to accept patentability of any of the enclosed requests, we request that the Board exerts its discretion to allow further claim amendments.

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If the Board does not see itself in a position to comply with any of our claim requests on the sole basis of our written submissions, we request oral proceedings in accordance with Article 116(1) EPC.

An adapted description can be provided at short notice.

I. MAIN REQUEST

1. INVENTIVE STEP (Article 56 EPC)

1.1. The Opposition Division (OD) concluded in section 2.27 of the contested decision that the skilled person is taught by D5 to reduce the dosage interval to solve the problem of complement breakthrough in paroxysmal nocturnal hemoglobinuria (PNH) patients receiving the priming dosage described in D6. The OD took the position that the skilled person would have reduced the interval "slightly", to 12 days to resolve the symptoms, as allegedly suggested by D5.

The OD stated by referring to T1979/09 that the replacement of one dosing regimen with another is a matter of "routine experimentation", regardless of the nature of the agent(s) being administered, the condition being treated, the relevant patient population or the complexity of the dosing regiment being "replaced".

1.2. Contribution of the invention to the art

Claim 1 of the main request is directed to eculizumab for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein eculizumab is to be administered by intravenous infusion to the patient under the following dosing schedule:

(i) 600 mg each week for four weeks;
(ii) 900 mg 1 week later;
(iii) 900 mg on a biweekly basis; and thereafter
(iv) 900 mg every 12 days,

wherein said patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii).

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Hence, the claims of the main request cover a specific and narrow four-stage treatment schedule for PNH patients with the anti-C5 antibody eculizumab.

As is well-known PNH is a rare, genetically acquired, life-threatening disease of the blood characterized by destruction of red blood cells by the complement system. The primary clinical manifestations of PNH range from hemolytic anemia, thrombosis or other deficiencies in hematopoiesis PNH patients may develop thrombosis (a blood clot) at some point in their illness, which is the main cause of severe complications and death in PNH. In fact, **about 50% of the patients die within 10 years** after diagnosis of the disease.

In 2007, eculizumab was approved for the treatment of PNH and dramatically changed the outlook for patients with PNH. Depending on the underlying extent of bone marrow failure certain patients can experience a breakthrough in hemolytic activity toward the end of the dosing interval (see discussion in paragraphs 76-78 of the patent). In order to ensure that PNH patients benefit from the treatment with eculizumab to the desired effect that **complement breakthrough is prevented** it is of utmost importance that eculizumab is administered in an appropriate dosage regimen.

The four-stage treatment schedule set forth in claim 1 of the main request is tailored to this appropriate dosage regimen of eculizumab. Eculizumab has the trade name Soliris®. The leaflet of Soliris® has been submitted as D15 during the opposition hearing. The claimed four-stage treatment schedule can be taken from sections 2.1 and 5.6 of D15.

The claims are therefore directed to the exact dosage regimen of eculizumab which saved lives of PNH patients and will do so in the future. These PNH patients have a decreased or even no need for blood transfusions, as well as a significantly decreased incidence of thrombosis. The contribution of the present invention to the art therefore should not be underestimated.

1.3. Difference between the closest prior art and the main claim

The claims of the main request provide for an improved treatment schedule of PNH patients showing complement breakthrough when previously treated using

the established dosage schedule (i.e. steps (i) to (iii) of claim 1). As D5 addresses the problem of complement breakthrough, D5 has been considered as closest prior art.

D5 is a conference abstract authored by inventors of the revoked patent. The following is an excerpt of D5:

> "We previously reported that eculizumab was very effective at acutely controlling intravascular hemolysis in a 3-month Pilot study of transfusion-dependent PNH patients. We now report that all 11 patients from the initial study entered an extension trial of 900 mg eculizumab by i.v. infusion over 30 minutes every other week for one year. [...] Two patients experienced transient breakthroughs in complement activity associated with LDH, hemoglobinuria and mild symptoms; these resolve immediately upon next dose of eculizumab and further breakthroughs were prevented by a slight decrease in dosing interval." (emphasis added)

Details on the 3-months pilot study as well as the slightest information on what is meant by a "slight decrease" are absent from D5. The difference between D5 and the claims is therefore two-fold:

(A) D5 is silent about the dosage schedule applied prior to the biweekly administration of 900 mg (i.e. steps (i) and (ii) of claim 1).

(B) D5 is silent about the feature of administering 900 mg every 12 days (step (iv) of claim 1 of the main request).

In view of D5 the technical problem underlying the claims may be seen as the provision of a complete eculizumab dosage regimen which is capable of preventing complement breakthroughs in all PNH patients, including those PNH patients which suffered from a complement breakthrough in the extension trial; see also section 2.15 of the impugned decision.

1.4. Distinguishing feature (A)

1.4.1. Two possible dosage schedules – both being applied prior to the biweekly administration of 900 mg (i.e. step (iii) of claim 1) - can be found in the prior art; see sections 2.18 and 2.19 of the impugned decision. While D6 describes a schedule which comprises steps (i) and (ii) of claim 1, D3 and D4 only describe

step (i) but not step (ii). The skilled person is consequently confronted with the necessity to choose from two options, being a two step or a one step dosing schedule prior to step (iii) of claim 1.

In its assessment of inventive step the OD found that the skilled person would have reverted to D6 as secondary reference, since only D6 but neither D3 nor D4 discloses a dosing schedule comprising the administration of (i) 600 mg eculizumab each week for four weeks, (ii) 900 mg eculizumab 1 week later and (iii) 900 mg eculizumab on a biweekly basis as set forth in claim 1. The OD justified its position that the skilled person would have preferably turned to D6, by stating that D6 allegedly describes "the most recent and detailed teaching"; see section 2.25 of the impugned decision.

1.4.2. The skilled person can be defined in the present case as a medical doctor, more specifically a clinician or a team of clinicians specializing in the treatment of haemological disorders.

The oral communication corresponding to the abstract of D3 has been presented in April 2003, the abstract D4 is from November 2002, and the abstract D6 dates September 2003. Thus, from the outset, all three references were published within the same one year period. D6 would not necessarily have been considered more recent in any meaningful way which would have led the skilled artisan to have favored this reference. Moreover, while D6 mentions step (ii) of claim 1, neither D3 nor D4 mentions this step. Hence, the teachings of D3 and D4 are consistent, while D6 teaches something else.

Importantly, D6 assigns no particular relevance to the presence or absence of step (ii). However, step (ii) 900 mg eculizumab 1 week later – is **essential** in order to provide an effective and optimal dosage regimen, as evidenced by section 2.1 of D15 (i.e. the post-filing product leaflet of Soliris®). Indeed, this could only be known through hindsight. In this respect reference is made to section 4 of the declaration D14. The technical expert Dr. Bell explains in section 4 that

> "This dose is important because, without it, the loading dose specified by step (i) does not provide an adequate baseline of complement inhibition that can be maintained by the maintenance dose of step (iii) or (iv)"

It follows that the omittance of step (ii) puts the PNH patients at an undesired increased risk of developing complement breakthrough.

In view of the contents of D3, D4 and D6 is not entirely clear whether D3 and D4 on the one hand and D6 on the other hand describe the same clinical study on PNH patients with eculizumab or two different studies. At face value and in view of the discussed differences there is some evidence that there are two different studies. However, even if considering that all three documents describe the **same** study all PNH patients would already have received the prior dosing schedule prior to the submission of the three abstracts by their authors. The OD's assumption that D6 describes something more recent and more detailed than D3 or D4 is therefore not correct under the assumption that the same study is described.

Irrespectively of whether assuming that the same clinical study or two different studies are described, the conclusion of the OD that the skilled person immediately and unquestionably knows that only D6 but not D3 or D4 describe the correct prior dosing schedule can only be drawn based on hindsight.

The OD in selecting D6 as a secondary reference obviously applied an "ex-post facto" analysis. As the Board is well aware, the Boards of Appeal have ruled that an "ex-post facto" approach when assessing inventive step is impermissible (see, for example, T 24/81, T 564/89, T 645/92 or T 795/93).

A correct application of the problem and solution approach has to avoid the inadmissible "ex-post facto analysis", the OD's biased selection of D6 as secondary reference already indicates that the decision to revoke the patent for an alleged lack of inventive step is not correct.

1.5. Distinguishing feature (B)

As it can be taken from the examples of the patent at paragraph [0073]

> "During the extension study, 2 patients did not sustain levels of eculizumab necessary to consistently block complement. [...] A reduction in the dosing interval from 14 to 12 days was sufficient to maintain levels of eculizumab

> above 35 µg/ml and effectively and consistently blocked serum hemolytic activity for the remainder of the extension study for both patients."

It will be detailed below that the OD's assumption that the skilled person in view of D5 – even if considering D6 as the "preferred" secondary reference - would have arrived at administering 900 mg every 12 days (step (iv) of claim 1 of the main request) without inventive skills is based on unjust oversimplifications.

1.5.1 *Optimizing the eculizumab dosing schedule is not a matter of routine*

First, there is nothing routine about treating PNH patients with eculizumab, which is a humanized monoclonal antibody that binds to complement protein C5 and prevents its cleavage into C5a and C5b.

As is well-known, the complement protein C5 is a plasma protein. In order to fulfill its function it must be present throughout the body and at a relatively high concentration of 75 µg/ml (0.4 µM) in normal serum; see paragraph [0031] of the patent.

The skilled person is particularly cautious when it comes to interfering with the complement system which is a key component of the human immune system. This is also expressed in D13:

> "Since complement activation has both positive and negative effects, the risk associated with complement inhibition is probably not negligible and long term studies will be necessary to assess possible risks. In particular, a theoretical concern is the role that C5 plays in hepatic regeneration and in combating granulomatous infections such as tuberculosis. Long term studies will determine whether C5 inhibition could have deleterious effects in these processes." (see page 1021; left hand column; top)

The skilled person would have correctly proceeded very cautiously when either increasing the dose or reducing the dose of eculizumab. For example, D15 carries a prominent "Black Box" warning at the top of the first page. The warning states the following:

> "Life-threatening and fatal meningococcal infections have occurred in patients treated with soliris and may become rapidly life-threatening or fatal if not recognized and treated early"

Furthermore, at the time the invention was made, those skilled in the art were fearful that treatment with eculizumab, which causes the PNH red cell clone size to increase from 36.7% to 58.9% could leave the patient vulnerable to a massive haemolytic attack if treatment was withdrawn (see paragraph [0069] of the patent). This fear is expressed in D7 as follows:

> "The reduction or cessation of transfusions and control of hemolysis in patients on eculizumab leads to an increase in the proportion of PNH red cells toward the level of PNH neutrophils [...] This raises the concern that stopping eculizumab for any reason might render the patient susceptible to a massive haemolytic attack and profound anemia." (see D7, page 53, lower left column).

Moreover, we refer to the sentence bridging pages 52 and 53 of D7, where it is stated:

> "The only apparent sequela [of treatment with eculizumab] is an increased risk of infection by encapsulated organisms, namely *Hemophilus influenza*, *Neisseria meningitides* and *N. gonorrhoea*." (information in brackets added)

As such, the skilled person would be highly concerned about the possibility of severe bacterial infection, and thus had manifest reasons to be exceptionally cautious in view of the specific facts of the present case, namely the potentially dangerous side effects of the anti-C5 antibody eculizumab.

As the Board is well aware, there is settled case law about the attitude of the skilled person in the field of life sciences. The skilled person is cautious and maintains a conservative attitude. She or he does not take unpredictable risks. This point applies to an exceptional extent and should be emphazised in the present case. Choices which carry minimal risk and thus might be considered worth trying or obvious to try (as they are less dangerous) in a particular context, cannot be equated with choices which carry potentially devastating, life threatening risks. The risks posed in the present case, involving a complex biologic drug, i.e. eculizumab, involved in a fundamental, life-depending pathway would have required a significantly heightened evaluation of the potentially available options.

This point is highlighted by a different drug of the same class, alemtuzumab, a humanized anti-CD52 antibody which was first approved in 2001 for refractory B-cell chronic lymphocytic leukemia (B-CLL). Alemtuzumab has exceptional lympholytic activity, driven by complement activation antibody-dependent cytotoxicity, and apoptosis. Accordingly, it was well known that this drug often causes severe side effects, particularly when administered intravenously. For example, as reported at page 6 of the enclosed monograph (BC Cancer Agency Cancer Drug Manual, D16[1]) for alemtuzumab, intravenous administration can cause "serious and potentially fatal complications including myocardial infarction, cardiomyopathy, cardiac arrest, arrhythmias, bronchospasm, and acute respiratory distress". Thus, several pre-treatment protocols involving immunosuppressants are recommended prior to administering alemtuzumab, as well as starting with low doses of the drug (3 mg) and escalating the dosage while carefully monitoring patients. In fact, the subcutaneous route has been studied in an attempt to reduce side effects and make the treatment more manageable (see Hale et al. (2004) *Blood* 224(4):948-955 being enclosed herewith as D17[2]). Thus, like eculizumab, optimizing the dosage for alemtuzumab was anything but routine and is still being explored, even at dosages on a magnitude of 200 to 300 times lower than the presently claimed dosages for eculizumab.

D17 goes on to discuss how alemtuzumab may cause substantial "first dose" reactions as a result of cytokine release when it is given intravenously at 3,18 and 19 days. The standard treatment regimen involves dose escalation and frequent, relatively small doses. A full treatment course for resistant CLL consists of approximately one gram of the antibody (see page 954 of D17). This is in stark contrast to the presently claimed, far higher 600 mg and 900 mg dosages (0.9 g every two weeks) for eculizumab, which would have been considered to hold a much greater risk. Moreover, many factors can affect the immunogenicity of alemtuzumab, including the dose and length of treatment, the underlying disease, prior exposure to chemotherapy, concomitant immunosuppressive drugs, and route of administration or the formulation of the drug (see discussion on page 954 of D17). Again, this highlights the fact that optimizing the dosages of

[1] This document is only submitted for providing the Board with an example of a drug which caused fatal complications due to overdosing.
[2] This document is only submitted for providing the Board with an example of a drug which caused fatal complications due to overdosing.

complex, antibody-based therapeutics depends on a number of complex, varying factors, and was far from "routine" at the priority date of the present application, even at dosages far lower than those presently claimed.

Indeed, the authors conclude that alemtuzumab provides a useful therapeutic option for patients with CLL. It has excellent activity as first-line treatment and can give good responses in patients who are unresponsive to conventional chemotherapy. However, "the optimal dose route and regimen are not yet known" (see page 955 of D17).

When C5 regulation is perturbed as in PNH the results can be catastrophic. As discussed in paragraph 5 of the declaration D14 of the technical expert Dr. Bell and paragraphs [0002]-[0005] of the patent, failure to **completely** suppress C5 in PNH leads to hemolysis and thrombosis. As mentioned above, thrombosis is the leading cause of death in PNH patients and 50% of PNH patients die within 10 years.

Solving the problem of completely suppressing C5 requires the use of very large doses of eculizumab. However, modifying large doses of any biologic, especially to increase levels of the drug in a patient's serum (e.g., by increasing dosages or shortening dosing intervals), was a risky and dangerous proposition at the priority date, i.e. 2004, not to mention the added complexity in the case of eculizumab where complement C5 plays a **fundamental role in the human immune system**. At that time, and largely still today, the 900 mg maintenance dose was unique among biologic drugs. The dose for most biologic drugs is less than 300 mg. A typical example is the therapeutic anti-TNF antibody adalimumab which is administered at 40 mg every two weeks (see, for example, http://www.drugs.com/dosage/humira.html).

1.5.2. *Possible paths for optimization*

The contents of the prior art do not create a one-way street situation contrary to the position obviously taken by the OD. As mentioned, adjusting the dosing of a biological which interferes with the complement system was and is a most sensitive issue and cannot be equated with a routine measure. This is simply because there was no routine to be relied upon. D5 does not provide a complete

teaching how to prevent complement breakthrough in PNH patients because what a "slight decrease in dosing interval" means is entirely open. Hence, at the priority date different paths for optimization were available, such as a 13.5 day interval or 13 day interval. There was no pointer to a 12 days interval. Rather the skilled person would have shied away from such a significant decrease in the time interval.

In the case of the present invention, it was PK/PD modeling and data that led the inventors to identify the 12 days interval as being the **precise and critical** solution to the problem, among other available, potentially useful options. Paragraph [0078], Figures 4 and 8 of the patent exemplify the pharmacodynamic data driven analysis used to choose 12 days. It is again only through hindsight – i.e. starting from the work of the inventors and physicians conducting the eculizumab clinical trials - to assume that the skilled person could have known that the specific changes to the clinical protocol that resulted in the present claims were safe and highly effective at preventing breakthrough. Importantly, it was not obvious or possible to know in advance whether a dangerous inflammatory event would occur at the increased dose resulting from the shortened dose interval. Nor was it obvious or possible to know in advance whether these new conditions would trigger an increased human anti-drug antibody response. Only by way of inventive work resulting in the presently claimed invention (i.e., a highly specific and unpredictible change in dosing frequency among other options) was this discovered.

The fact that the inventors first performed labor intense and inventive PK/PD modeling before actually applying dosing step (iv) to PNH patients underscores how critical any dosing change of eculizumab is. It is in particular no option to use a "try-and-see" approach in PNH patients, given that such an approach would involve irresponsible risks for the PNH patients.

1.5.3. *D5 fails to render decreasing the dosing interval from 14d to 12d obvious*

Appellant strongly urges the Board to reconsider the patentability of the main request on the grounds of an alleged lack of inventive step. The OD has significantly erred in hinging its decision on the vague statement in D5 of "slightly decreasing" the dosing interval to support the assertion that the claimed invention

lacks inventive step. Indeed, the OD offers no basis whatsoever for its conclusion that the skilled person would have automatically equated a "slight decrease" in dosing interval with a decrease of considerably more than 10% (i.e., from 14 days to 12 days). Again, this is an ex-post facto approach.

A change from a 14 day interval to a 12 day interval for a drug administered at 900 mg for maintenance dosing results in a **significant increase** in exposure to the patients of 3.9 grams or **16.7%** of therapeutic antibody annually. The magnitude of this change is not commensurate with the phrase "slight decrease" as suggested in D5 (see section 2.20 of the contested decision) but would have carried great risks.

The OD decision should be reversed in this appeal, because the selection of the 12 day dosing interval for patients experiencing breakthrough required inventive skills. As discussed in the above-cited paragraph [0073] of the patent, the dose decision was made to maintain a minimum target level of 35 µg/ml of eculizumab in the affected patient's serum. This change was supported scientifically by examining the PK/PD data generated in patients with PNH and being treated with eculizumab. The OD's reliance on the statement in D5 trivializes the significance of this non-obvious and precise solution to a clinically devastating problem. As discussed above, the change of dosing interval from 14 days to 12 days to prevent breakthrough was not made by simple process of elimination or a try-and-see approach, but rather by inventive reasoning and a carefully selected scientific approach.

1.5.4. *Decision T1979/09 is not applicable*

In addition, we wish to submit that analogizing the decision T1979/09 to the facts and circumstances of the present patent is incorrect. The claims at issue in T1979/09 were directed to a dietary supplement for preventing or treating discomfort associated with venous insufficiency by administering flavonoids by water extracting crushed and dried red vine leaves. This case is distinguishable from the present matter and the decision states that

> "the skilled person would carry out these steps according to the known standard technologies for the processing of medicinal plants" (see page 17 of T1979/09).

It cannot be the case that dose considerations made in the context of suppressing complement protein C5 by administering a high dose of a biologic drug to patients with a potentially fatal disease by intravenous administration are routine experimentation on the level of administering flavonoids extracted from leaves of medicinal plants. The consequences of making a mistake in either of the two situations are certainly in no way equivalent.

Cleavage of complement C5 plays a central role in the immune system; see Rother et al. *Nature Biotechnology* 25 (11) 1256-1264 (2007) which is provided herewith as document D18[3]. There are three distinct complement pathways known as alternative pathway, classical pathway, and lectin pathway and they differ in the mechanism of activation. But no matter the manner of activation, all three pathways converge at the point of C5 cleavage. C5 is cleaved into C5a and C5b. C5a is a potent anaphylatoxin that mediates leukocyte chemotaxis, increases vascular permeability and induces other secondary inflammatory mediators. The simultaneous release of C5b triggers the recruitment of terminal complement components C6, C7, C8 and C9 to form the terminal complement complex which results in lysis and destruction of a target cell. Individuals who are deficient in other complement components such as C1, C2 or C4 develop severe autoimmune symptoms such as Lupus-like syndrome and are at recurrent risk of developing sepsis. Therefore, the complete inhibition of complement C5 was rightly viewed in 2004 as a very uncertain and medically consequential activity.

According to the European Medicines Agency (EMA) in their Assessment Report on *Vitis vinifera* L.

> "Since ancient times, beneficial effects on health have been ascribed to wine and vine leaves, as confirmed by numerous "recipes" reported in Egyptian papyri, the Sumerian Tablets, the writings of Hippocrates of Cos (5th-4th Century BC), Celcus (1st century AD), Galen (130-201 AD) and Paracelsus (1493-1541). (see EMA/HMPC/16633/2009 section 2, Historical Data on Medicinal Use provided herewith as document D19[4]).

[3] This document is only submitted for providing the Board with a short and concise overview of the scientific background of the invention.
[4] This document is only submitted for providing the Board with background information on the case at stake in T1979/09.

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The incorporation of the flavonoids into in a dose range of 300-800 mg and incorporated into a dietary supplement tablet does not change the fact that the flavonoids were known to be generally safe and beneficial. The flavonoids were orally administered and allowed to diffuse into the blood stream in the digestive process. The EMA found that for red vine leaf converted from a tea administration to a tablet

> "there is no safety specific information that is relevant for medical products." (see EMA/HMPC/16633/2009 section 5.3, Adverse Events).

It follows that the administration of flavonoids has no severe side effect and overdosing flavonoids in a patient is not life-threatening for the patient. By contrast, overdosing the first in class biologic protein drug eculizumab may result in the blocking the complement system of the immune system to an extent that puts the patient in a life-threatening situation. In the first case optimizing of the dosing schedule is a matter of routine, whereas in the latter case it is not.

Thus, we contend that dosing through intravenous administration of a first in class biologic protein drug, at an usually high dose, with the intention of suppressing the activity of an immune system component does involve inventive activity. Where the consequences of dosing changes may be catastrophic, the inventors must invest greater judgment and thought about dose changes or dose interval changes. The inventors for a drug such as eculizumab are evaluating the effects of dose changes on the immune system in a very quantitative fashion. Any statements made in the context of dosing decisions for dietary supplements should not apply with equal force to the clinical trials of a first in class drug like eculizumab. We therefore consider that T1979/09 is not applicable. The facts of the case underlying this decision are different from that of the present case.

1.6. In summary, we submit that the reasons of the OD for revoking the patent for an alleged lack of inventive step are without merit. The Board of Appeal is therefore requested to set the decision of the OD aside and to give the order to maintain the initially granted patent based on the main request.

II. AUXILIARY REQUEST 1

In case the Board of Appeal cannot accept that the main request meets the requirements of Article 56 EPC, consideration of auxiliary request 1 is requested. The arguments and facts presented herein above in connection with the main request are equally applicable to the auxiliary request 1.

As compared to the main request claim 1 of auxiliary request 1 further comprises the feature of "reducing cell lysis to below 20% of normal serum complement activity" which is a feature indicating the effective inhibition of haemolytic activity; see page 38, lines 11-13 and Figure 8. The effective inhibition of haemolytic activity is a key advantage being associated with the subject-matter claimed in the main and auxiliary requests. This advantage is expressly incorporated in the main claim of auxiliary request 1.

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Enclosure:
D16 (Monograph from the BC Cancer Agency Cancer Drug Manual)
D17 (Hale et al. (2004) *Blood* 224(4):948-955)
D18 (Rother et al. *Nature Biotechnology* 25 (11) 1256-1264 (2007))
D19 (EMA/HMPC/16633/2009 section 2, Historical Data on Medicinal Use)
Main Request
Auxiliary Request 1

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(05 71 2607.0 / 1720571)
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Our Ref.: M2367 OPP(EP)

PERSPECTIVE

nature biotechnology

Discovery and development of the complement inhibitor eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria

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The complement system provides critical immunoprotective and immunoregulatory functions but uncontrolled complement activation can lead to severe pathology. In the rare hemolytic disease paroxysmal nocturnal hemoglobinuria (PNH), somatic mutations result in a deficiency of glycosylphosphatidylinositol-linked surface proteins, including the terminal complement inhibitor CD59, on hematopoietic stem cells. In a dysfunctional bone marrow background, these mutated progenitor blood cells expand and populate the periphery. Deficiency of CD59 on PNH red blood cells results in chronic complement-mediated intravascular hemolysis, a process central to the morbidity and mortality of PNH. A recently developed, humanized monoclonal antibody directed against complement component C5, eculizumab (Soliris; Alexion Pharmaceuticals Inc., Cheshire, CT, USA), blocks the proinflammatory and cytolytic effects of terminal complement activation. The recent approval of eculizumab as a first-in-class complement inhibitor for the treatment of PNH validates the concept of complement inhibition as an effective therapy and provides rationale for investigation of other indications in which complement plays a role.

The complement system: a potential target for therapeutics

The complement system consists of more than 20 serum proteins that interact in a precise series of enzymatic cleavage and membrane binding events leading to the generation of products with immunoprotective, immunoregulatory, proinflammatory and cytolytic properties[1,2].

Proximal complement. The cascade proceeds through three distinct pathways—alternative, classical and lectin—which all result in the generation of C3 convertase complexes that mediate the cleavage of C3 to C3a and C3b[1,3] (**Fig. 1**). Recent studies have delineated the structure of C3 and C3 fragments and provided important insights into their function[4–7]. The classical pathway is generally initiated by the interaction of C1q with antigen-antibody complexes. The lectin pathway is activated by the interaction of mannose-binding lectin (MBL) with specific mannose-containing carbohydrate structures. The alternative pathway is initiated by deposition of preformed C3b on a variety of substrates such as bacteria and cell membranes, including erythrocytes. This C3b is continuously available due to the interaction of C3 with water, a process that represents an important part of the innate immune system called complement 'tick-over'. In addition to its role in the initiation of alternative pathway activation, C3b is also necessary for the amplification and progression of the complement cascade through all pathways of activation and serves as a key immunoprotective and immunoregulatory molecule.

The importance of proximal complement components in immunity is illustrated by the clinical effects observed in people with genetic complement deficiencies. For example, individuals with C3 deficiency are at particular risk of infection from polysaccharide-coated bacteria such as *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Neisseria meningitidis*, and frequently die at a young age from overwhelming sepsis[8]. The absence of C3 is generally thought to prevent the progression of complement through all pathways of activation, thereby reducing the body's ability to opsonize pathogens and mount an inflammatory response, although some studies suggest that C5 cleavage can occur in the absence of C3 by thrombin[3,9]. Individuals deficient in C1, C2 or C4 develop severe autoimmune symptoms such as a lupus-like syndrome and are also susceptible to recurrent overwhelming sepsis. Thus, deficiencies in early components of the complement cascade lead to impairment in the ability to generate C3b, which is critical for immune complex clearance and is the primary opsonin for many pathogenic microorganisms.

Terminal complement. All pathways of complement activation converge at the cleavage of C5 into C5a and C5b by the C5 convertase enzyme complexes. This cleavage event initiates the terminal complement cascade (**Fig. 1**). C5a is a potent anaphylatoxin that mediates leukocyte chemotaxis, increases vascular permeability, alters smooth muscle tone and induces secondary inflammatory mediators such as hydrolytic enzymes, reactive oxygen species, arachidonic acid metabolites and cytokines[10]. C5a receptors are present on a variety of cell types that directly contribute to inflammation, including monocytes, macrophages and neutrophils. The C5a receptor CD88 is a member of the N-formyl peptide receptor family, which transduces signals through a G protein–dependent pathway resulting in biochemical proinflammatory

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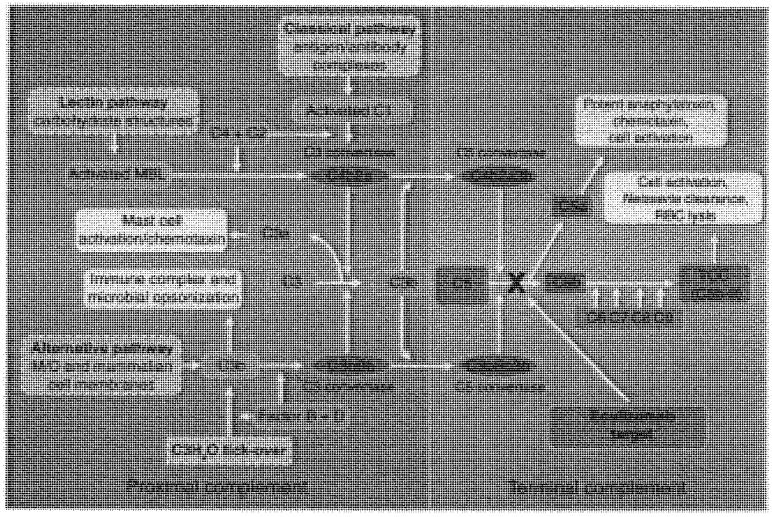


Figure 1 Targeted blockade of complement protein C5. The complement cascade can be activated via the classical, lectin and alternative pathways. The proximal components of complement (proteins upstream of C5) are essential for microbial opsonization and immune complex clearance. All pathways of complement activation converge at the cleavage of the terminal complement protein C5 leading to the generation of molecules with proinflammatory and cell lytic properties. Targeted blockade at C5 with eculizumab therefore prevents the deleterious properties of terminal complement activation while preserving the immunoprotective and immunoregulatory functions of proximal complement. M/O, microorganisms. TCC, terminal complement complex.

responses[11]. A second C5a receptor has been identified (C5L2) that also facilitates C5a-mediated signaling in a variety of cell types[12]. C5b recruits the terminal complement components C6, C7, C8 and C9 to form C5b-9 or the terminal complement complex (TCC) on the surface of cells. The generation of the TCC at sublytic concentrations stimulates the release of many of the same proinflammatory molecules described for C5a. Most importantly, unimpeded assembly of the TCC on the cell surface results in cell lysis. Deposition of TCC on erythrocytes results in the destruction of these cells in hemolytic diseases such as PNH[13,14].

The importance of terminal complement components in immunity is demonstrated by individuals with genetic deficiencies of C5, C6, C7, C8 or C9, who show an increased incidence of infections with *N. meningitidis*, a bacterium that can cause meningitis[8]. Vaccines against *N. meningitidis* are recommended for terminal complement–deficient individuals, although breakthrough infections have been reported[15]. Interestingly, *N. meningitidis* infections in terminal complement–deficient people are typically less severe than in normal individuals, possibly due to the lack of TCC-mediated endotoxin release from the bacteria[15].

Targeted blockade of the complement cascade at C5. In the rational design of a therapeutic complement inhibitor, C5 is an attractive target[1]. Because C5 is common to all pathways of complement activation, blockade at this point stops the progression of the cascade regardless of the stimuli. In addition, prevention of C5 cleavage effectively blocks the generation of the potent proinflammatory molecule C5a and the cell lytic TCC. Importantly, C5 blockade preserves the critical immunoprotective and immunoregulatory functions of upstream components that culminate in C3b-mediated opsonization and immune complex clearance.

Development of eculizumab

To generate potent inhibitors of complement component C5, panels of murine anti-human C5 monoclonal antibodies were generated and initially screened for the ability to inhibit TCC-mediated lysis of antibody-sensitized chicken erythrocytes by human complement in a standard hemolytic assay[16]. The most potent candidates were cloned and purified, and functional activity was further characterized to identify anti-C5 antibodies that could also effectively block the generation of C5a. Of ~30,000 hybridomas screened, one monoclonal antibody (mAb), designated murine 5G1.1 (m5G1.1 mAb), was identified that effectively blocked both TCC-mediated hemolysis and the generation of C5a, at a 0.5:1 molar ratio of antibody to C5. Murine 5G1.1 mAb binding to human C5 mapped to the N-terminal region of the alpha chain.

Engineering. To reduce the potential for immunogenicity, we cloned and grafted the complementarity-determining regions of m5G1.1 mAb into human heavy and light chain antibody frameworks (**Fig. 2**)[16,17]. The human heavy-chain framework was derived from the H20C3H antibody variable region[18], which contained no amino acid changes relative to the original germline sequence. The human light-chain framework was derived from the I.23 antibody variable region[19], which contained only one amino acid change from the original germline sequence; the germline amino acid residue was restored at this position. The use of purely germline framework acceptor sequences should further minimize potential immunogenicity of the humanized antibody, because the immune system of every individual should be tolerant to these sequences.

The use of antibodies as therapeutics in some clinical settings is complicated by effector functions of such antibodies that serve to activate complement and/or to bind to antibody receptors (Fc receptors) on inflammatory cells after they have engaged their target. To reduce the potential for eculizumab to elicit proinflammatory responses, we replaced the heavy-chain constant region of the parental antibody with components of both human IgG2 and IgG4 constant regions (**Fig. 2**)[20]. The human IgG2 antibody isotype does not bind Fc receptors[21,22], whereas the human IgG4 isotype does not activate the complement cascade[23,24]. The IgG2/IgG4 hybrid constant region of eculizumab includes the CH1 and hinge regions of human IgG2 fused to the CH2 and CH3 regions of human IgG4, and lacks the ability to bind Fc receptor and to activate complement[20]. To avoid the generation of an antigenic site during the fusion, we used a restriction endonuclease cleavage site common to both IgG2 and IgG4 to join the two constant regions. Indeed, a total of 31 amino acids flanking the fusion site are identical between IgG2 and IgG4.

Binding characteristics. The humanized monoclonal antibody eculizumab maintained its ability to block the cleavage of C5 during complement activation as the antibody potently inhibited both the generation of C5a and C5b-9–mediated human serum hemolytic activity[16]. Eculizumab is highly species-restricted, with minimal activity against any other primate or mammalian C5. No nonspecific or unexpected

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binding of eculizumab to any human tissue examined in a tissue cross-reactivity study was observed. In addition, the affinity of eculizumab for human C5 (K_d = 120 pM) is similar to that of the parental m5G1.1-mAb, indicating that the binding properties of the antibody were not compromised during the humanization process.

The eculizumab pharmacokinetic profile was analyzed using a one-compartmental model. When administered by intravenous infusion, eculizumab has a half-life of 272 ± 82 h, and its distribution appears to be primarily limited to the vascular space. Eculizumab serum concentrations appear to reach steady state after ~57 days. At steady state, the eculizumab accumulation ratio (R_{acc}) was calculated to be 1.075, which is indicative of minimal accumulation over time. Pharmacodynamic activity correlates directly with eculizumab serum concentrations and maintenance of trough levels above 35 μg/ml results in essentially complete blockade of hemolytic activity *in vivo*[25].

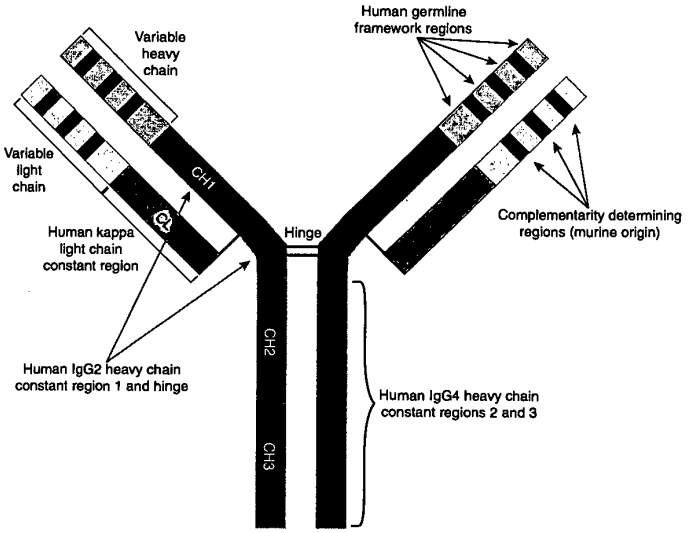


Figure 2 Engineering of eculizumab to reduce immunogenicity and eliminate effector functions. To minimize immunogenicity, we grafted murine complementarity-determining regions into human heavy and light chain germline antibody framework sequences. Additionally, human IgG2 and IgG4 heavy chain sequences were combined to form a hybrid constant region that is unable to bind Fc receptors or to activate the complement cascade. Eculizumab exhibits high affinity for human C5, effectively blocking its cleavage and downstream proinflammatory and cell lytic properties.

Preclinical development. Because of the species-restricted nature of eculizumab, pharmacological activities *in vivo* were explored with a surrogate anti-mouse C5 antibody (BB5.1) that demonstrated terminal complement–inhibitory activity comparable to that of eculizumab. As no adequate mouse models of PNH exist, *in vivo* studies targeting other therapeutic indications with potential involvement of terminal complement activation were used. In a collagen-induced model of arthritis, BB5.1 was shown to prevent the onset of disease and to improve the course of previously established disease[26]. Treatment with BB5.1 also resulted in an improvement in renal disease and in dramatic prolongation of survival in a lupus-like autoimmune model[27]. These models provided substantial evidence that a functionally blocking anti-C5 antibody could effectively and consistently block terminal complement activation leading to a reduction in terminal complement-mediated proinflammatory sequelae. The surrogate BB5.1 antibody was also used in mouse toxicity studies in which no compound-related mortalities or significant abnormalities were observed.

Early clinical development. Initial human clinical studies of eculizumab, and a single-chain antibody variant of eculizumab called pexelizumab, were performed in individuals with rheumatoid arthritis and systemic lupus erythematosus[28,29] and in individuals with coronary artery bypass graft surgery and myocardial infarction, respectively[30,31]. The initial eculizumab studies served two purposes, to determine a dosing schedule for future trials and to establish initial safety of terminal complement inhibition in humans. Participants received a single dose of eculizumab ranging from 0.1 mg/kg to 8 mg/kg; all doses were observed to be safe and well-tolerated. The 8 mg/kg dose (~600 mg total) provided complete complement blockade for 7–14 days. Studies exploring multiple chronic dose regimens with eculizumab were also performed in individuals with membranous nephritis and rheumatoid arthritis[32,33].

PNH—a disease in search of a complement inhibitor

Etiology of PNH. PNH is a rare form of hemolytic anemia with an estimated incidence of 1.3 cases per million per year and a prevalence of 15.9 cases per million[34]. The disease is manifest by an acquired genetic deficiency of endogenous complement inhibitors on the surface of blood cells; these cells are referred to as the PNH clone(s). PNH clones are deficient in proteins that are normally linked to the cell surface by a glycosylphosphatidylinositol (GPI)-anchor. The inability of cells to attach proteins through this lipid moiety is caused by somatic mutations occurring in the X-linked gene, phosphatidylinositol glycan–complementation class A (*PIGA*)[35–37], a critical molecule in the GPI-anchoring pathway.

In individuals with PNH, cells deficient in GPI-anchored proteins exist in all blood lineages including erythroid, myeloid and lymphoid cells. These PNH cells are clonal and harbor matching *PIGA* mutations indicating that the mutations occur in multipotent hematopoietic stem cells[38,39]. Granulocytes from the blood of healthy controls also possess *PIGA* mutations at an exceedingly low frequency (~1 in 50,000), suggesting that random mutations in this gene are important in the pathogenesis of PNH[40]. Thus, it has been hypothesized that the development of PNH requires two independent events to occur; hematopoietic stem cells must acquire a *PIGA* mutation and an immune attack that preferentially targets normal stem cells must be present[41,42]. This proposal presumes that the bone marrow insult targets a GPI-anchored protein on the hematopoietic stem cell, thereby providing a survival advantage to GPI-anchoring–deficient stem cells (that is, cells possessing a *PIGA* mutation). It should be noted however that *PIGA* mutations characterized in healthy controls appear to occur in more differentiated colony-forming cells rather than multipotent hematopoietic stem cells[39]. These colony-forming cells would not have self-renewal capacity and therefore would not be propagated beyond their terminally differentiated progeny.

There is clinical evidence to support a survival advantage of *PIGA* mutated hematopoietic stem cell clones over normal stem cells in damaged bone marrow. The mAb alemtuzumab (Campath) is used to treat

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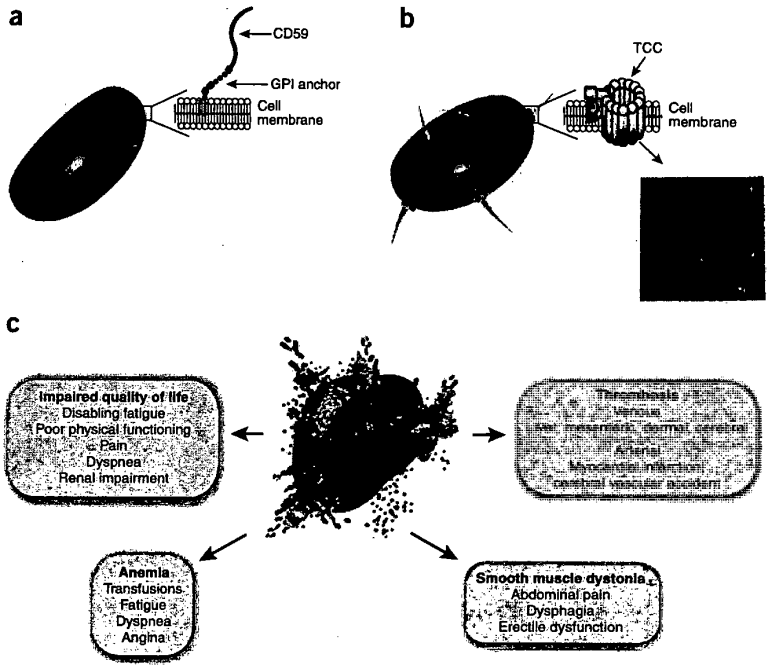


Figure 3 Terminal complement deposition on the surface of PNH RBCs results in chronic hemolysis and serious clinical morbidities. (**a**) Normal RBCs express complement regulatory proteins including the terminal complement inhibitor CD59. CD59 is tethered to the RBC lipid bilayer of the cell membrane by a GPI anchor. The presence of CD59 protects these cells from autologous complement-mediated destruction. (**b**) PNH RBCs lack GPI-anchored proteins including CD59. Continuous complement activation leads to formation of the terminal complement complex (TCC) that transverses the lipid bilayer. These complexes form numerous pores in the RBC membrane, as shown by the electron micrograph (inset). As pores form in the membrane, water enters the RBC (arrows), resulting from osmotic pressure. (**c**) As the concentration of TCCs increases, the PNH RBC swells and ultimately ruptures (hemolysis). Intravascular hemolysis causes the release of the RBC contents such as hemoglobin into the plasma. Hemolysis in PNH is chronic and leads to multiple clinical sequelae, including severe anemia requiring transfusions, disabling fatigue, dyspnea, impaired quality of life, recurrent pain associated with smooth muscle dystonia, and thrombosis. Artwork by Edmond Alexander, ©2007 Edmond Alexander, printed with permission. Micrograph image is from E.R. Podack. Molecular mechanisms of cytolysis by complement and cytolytic lymphocytes. *J. Cell. Biochem.* **30**, 133–170 (1986). Copyright © 1986 Wiley-Liss. Reprinted with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.

chronic lymphocytic leukemia by targeting and destroying malignant T- and B-cells that express the GPI-anchored protein CD52. In many individuals, a PNH T-cell clone expands after alemtuzumab treatment. In this case alemtuzumab supplies the bone marrow insult and the GPI-anchoring deficient progenitor T-cells that lack CD52 escape immune destruction and expand[43].

Pathophysiology of PNH. Although PNH blood cells of all lineages are missing the wide array of GPI-anchored proteins from their surface, they are remarkably normal with regard to their physiological functions (e.g., fighting infection, clotting, carrying oxygen). However, the PNH red blood cell (RBC) is exquisitely sensitive to destruction because of the inability to regulate autologous terminal complement assembly (**Fig. 3**). This defect is primarily triggered by the absence of terminal complement inhibitor function[13,14,44,45]. CD59 normally blocks formation of the TCC on the RBC surface, thereby preventing intravascular hemolysis[46–48]. Hemolysis in PNH is chronic because of a continuous state of complement activation (tick-over; **Fig. 1**), but brisk episodes of hemolysis (paroxysms) coincident with increases in complement activation triggered by infections, surgery, strenuous exercise, excessive alcohol intake and blood transfusions frequently occur[49].

Chronic hemolysis is central to the morbidities in PNH and contributes to mortality in those with the disease (**Fig. 3**). It may result in severe anemia requiring transfusion, and refractory transfusion-dependent hemolytic anemia has been considered a major complication of PNH warranting consideration of bone marrow transplantation[35]. Fatigue in PNH patients is tied directly to hemolysis, strikingly disproportionate to the degree of anemia, frequently debilitating and similar to the severity observed in anemic cancer patients[25,49–52]. Hemolysis is also directly linked to diminished health status and functioning[25,49]. Other disabling morbidities evidenced by individuals with PNH that are likely a direct result of hemolysis include dysphagia, abdominal pain, erectile dysfunction, pulmonary hypertension and renal failure[49,53,54]. Thromboembolism, the most feared complication in PNH, is also directly associated with hemolysis and may be induced by the release of free hemoglobin, consumption of nitric oxide, and subsequent clotting[53,55–63].

Thrombosis is clinically evident in ~40% of individuals with PNH and is the leading cause of premature mortality[64–68]. Although venous thrombosis is more typical in PNH, arterial thrombosis, particularly in the cerebral circulation, is not uncommon[56,64]. Thrombosis is evident in individuals with small and large PNH clone sizes and varying degrees of hemolysis, although it appears to be more common in those with larger clones[56,57,66,68]. Individuals with hemolysis but without a history of transfusion also experience thrombosis, including fatal first thrombosis[57]. Subclinical thrombosis has been reported in 60% of a cohort of PNH patients, despite use of anticoagulation, and unrelated to transfusion requirements[69].

Complement inhibitors in PNH. As PNH is defined by the acquired genetic deficiency of the endogenous terminal complement inhibitor CD59 on the surface of blood cells, restoration of terminal complement inhibition should effectively reduce hemolysis in PNH and abrogate the serious clinical morbidities associated with this life-threatening disease. Initial attempts to circumvent the functional defect in PNH cells focused on replacing CD59 on the surface of PNH cells through gene therapy. A recombinant, transmembrane form of CD59 (CD59-TM) was generated and analyzed for the ability to regulate complement activity[70]. A GPI-anchoring deficient complement-sensitive B-cell line derived from an individual with PNH was virally transduced with CD59-TM, resulting in protection against classical complement-mediated membrane damage. These data established that a functional transmembrane form of CD59 can be expressed on the surface of PNH cells

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through a gene therapy approach. Retroviral transduction of PNH erythroid cells with a functional *PIGA* gene has also been shown to restore GPI-anchor protein expression on the surface of these cells, thereby protecting them from complement-mediated destruction[71]. However, to provide a renewable source of complement-protected erythrocytes in individuals with PNH through a gene therapy approach, efficient targeting of early erythroid progenitor cells would be required, which has not been achieved to date. This led to the development of the complement inhibitor eculizumab, which binds to and blocks the cleavage of C5 in plasma, thereby reestablishing terminal complement regulation in the plasma of individuals with PNH. The potential effect of terminal complement blockade on hemolysis in PNH was observed in a person with coexistent C9 deficiency and PNH who had no signs of hemolysis[72]. PNH was diagnosed in this individual only after the administration of a whole blood transfusion during a routine surgery, which served to reconstitute C9 and trigger hemolysis.

Table 1 Efficacy results of the open-label phase 2 pilot study of eculizumab in individuals with PNH

	Before treatment	During eculizumab	P value
Units of packed RBCs transfused/patient/month			
Median	1.8	0	0.003
Mean ± s.e.m.	2.1 ± 0.83	0.6 ± 0.67	
LDH (mean ± s.e.m., U/L)*	3111 ± 568	594 ± 32	0.002
PNH RBCs (proportion)	36.7 ± 5.9	59.2 ± 8.0	0.005
Days with hemoglobinuria/patient/month	2.9	0.12	<0.001

*The normal range for LDH in this study was 150–450 U/L.

Eculizumab in PNH: clinical trials

Eculizumab was evaluated in PNH in three separate parent studies—a phase 2 pilot study and two phase 3 studies. All participants in these trials were allowed to continue eculizumab in follow-on extension studies.

Pilot study. The first eculizumab PNH study (beginning in May 2002) evaluated terminal complement inhibition in a 3-month open-label phase 2 pilot study in 11 participants with hemolytic PNH at two sites in the United Kingdom[73]. In this study, participants with at least four transfusions in the previous 12 months received 600-mg infusions of eculizumab every week for 4 weeks, followed 1 week later by a 900-mg dose and then by 900 mg every other week through week 12. In this study and all subsequent trials, participants were vaccinated against *N. meningitidis* at least 2 weeks before the first dose of eculizumab. Clinical and biochemical indicators of hemolysis were measured throughout the trial. Mean lactate dehydrogenase (LDH) levels decreased from 3111 U per liter before treatment to 594 U per liter during treatment ($P = 0.002$; **Table 1**). The mean percentage of PNH erythrocytes increased from 36.7% of the total erythrocyte population to 59.2% ($P = 0.005$). The mean and median transfusion rates decreased from 2.1 and 1.8 units per individual per month to 0.6 and 0.0 units per individual per month, respectively ($P = 0.003$, for the comparison of the median rates). Episodes of hemoglobinuria were reduced by 96% ($P < 0.001$), and measurements of the quality of life substantially improved. From this study, it appeared that eculizumab was safe and well-tolerated in individuals with PNH. This terminal complement inhibitor reduced hemolysis, hemoglobinuria, and the need for transfusion, with an associated improvement in quality of life.

After completion of the initial 12-week study, all 11 participants from the initial PNH study chose to continue treatment in a 52-week extension study; all participants completed the study[25]. The dramatic reductions in hemolysis and the need for transfusion that were observed in the pilot study were maintained throughout the extension study, and significant improvements in quality-of-life measures also continued. In this study, long-term eculizumab administration continued to be safe and well-tolerated. Clinical breakthrough occurred in two individuals with insufficient eculizumab levels; shortening the dosing interval (increasing the total dose) resulted in rapid restoration of complement suppression, abrogation of hemolysis, and resolution of disease signs and symptoms. These data demonstrate the close relationship between sustained terminal complement inhibition, hemolysis, and symptoms in individuals with PNH. Further, dramatic clinical improvements in signs and symptoms attributed specifically to hemolysis including hemoglobinuria, and the smooth muscle dystonias—abdominal pain, dysphagia and erectile dysfunction—were observed in these participants[54]. Ten of the original 11 participants have remained on eculizumab for more than 5 years of continuous treatment.

Phase 3 pivotal program. The results from the initial open-label phase 2 pilot study were sufficiently compelling that discussions were initiated with the FDA and the EMEA in order to plan phase 3 studies for registration in the United States and Europe, respectively. In 2003,

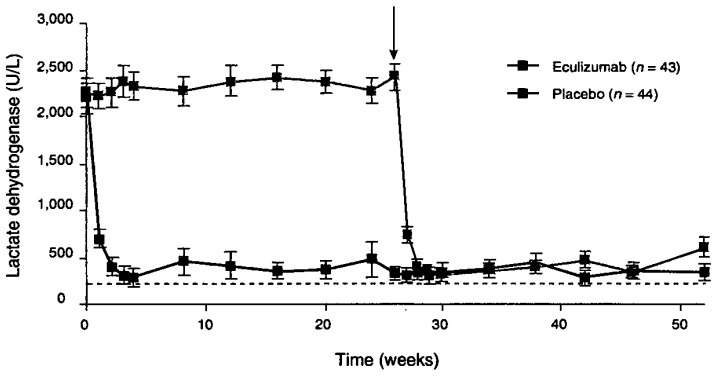


Figure 4 Reduction in hemolysis during treatment with eculizumab. Mean levels of lactate dehydrogenase reflect the degree of hemolysis from baseline to week 52. The dashed line indicates the upper limit of the normal range for lactate dehydrogenase (normal range, 103 to 223 U per liter). In eculizumab-treated patients, the mean level of lactate dehydrogenase was rapidly reduced to just above the upper limit of the normal range. In the placebo group, the mean level of lactate dehydrogenase remained highly elevated. The arrow depicts the transition of placebo-treated patients in TRIUMPH to eculizumab treatment in the phase 3 extension study at which time levels of lactate dehydrogenase rapidly reduced to near normal values.

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Table 2 Primary and secondary endpoints of the phase 3 pivotal efficacy study TRIUMPH

	Treatment period		
	Placebo	Eculizumab	*P*-value
Stabilization of hemoglobin levels (percent of patients)[a]	0	49	<0.000001
Units transfused/patient[a]			
Median	10	0	<0.000001
Mean ± s.e.m.	11.0 ± 0.83	3.0 ± 0.67	
Transfusion Avoidance (percent of patients)	0	51	<0.000001
FACIT-Fatigue score (change from baseline)	−4.0 ± 1.7	6.4 ± 1.2	0.000006
LDH (area under the curve; U/L × day)	411,822	58,587	<0.000001

[a]Co-primary endpoints

eculizumab received orphan designation for PNH in both the United States and Europe. The phase 3 eculizumab in PNH program consisted of: TRIUMPH, the pivotal trial that examined eculizumab safety and efficacy in a hemolytic PNH population with at least 4 transfusions in 12 months; SHEPHERD, which examined safety and efficacy of eculizumab in a broader and more heterogeneous hemolytic PNH population in individuals with a history of transfusion; and a phase 3b extension study to evaluate long-term safety and efficacy, including the potential anti-thrombotic effect of eculizumab.

TRIUMPH was a double-blind, randomized, placebo-controlled, multicenter, phase 3 trial[74]. Individuals were first enrolled and treated in 2004 and received either placebo or eculizumab intravenously; eculizumab was given at a dose of 600 mg weekly for 4 weeks, followed 1 week later by a 900-mg dose and then 900 mg every 14 ± 2 days through week 26. Eighty-seven participants (43 eculizumab, 44 placebo) at 34 international sites were enrolled. The two primary endpoints were the stabilization of hemoglobin levels and the number of units of packed red cells transfused. Biochemical indicators of hemolysis and the participant's fatigue and quality of life were also assessed. After enrollment, participants were observed for up to 13 weeks. Those who did not require a transfusion during the observation period were considered ineligible for randomization. Eighty-seven individuals underwent randomization. Every participant treated with eculizumab experienced an objective response. Across all treated participants, a significant reduction in hemolysis (as measured by LDH) was observed after 1 week of treatment and maintained throughout the 26 week study in the eculizumab group but not in placebo (**Fig. 4**). There was an 85.8% lower median area under the curve for LDH plotted against time (in days) in the eculizumab group, as compared with the placebo group (58,587 versus 411,822 U per liter, respectively; $P < 1 \times 10^{-6}$; **Table 2**). After completion of the study, a similar reduction in hemolysis was demonstrated with placebo participants transitioning to eculizumab. Stabilization of hemoglobin levels in the absence of transfusions was achieved in 49% (21 of 43) of the individuals assigned to eculizumab and none (0 of 44) of those assigned to placebo ($P < 1 \times 10^{-6}$). During the study, a median of 0 units of packed red cells was administered in the eculizumab group, as compared with 10 units in the placebo group ($P < 1 \times 10^{-6}$). A total of 51% of the eculizumab group remained transfusion independent during the entire 26-week study, whereas no one in the placebo group achieved this status. Even among participants receiving eculizumab in whom transfusion independence was not achieved, transfusion requirements were reduced by 44% as these individuals also demonstrated a clinically and statistically significant reduction in hemolysis. The reduced but continued requirement for transfusion support in some individuals may reflect more severe bone marrow aplasia and/or residual low-level hemolysis through the extravascular compartment[74]. Clinically and statistically significant improvements were also found in fatigue, as measured by scores on the Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT-Fatigue) instrument ($P = 6 \times 10^{-6}$) and the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire (EORTC QLQ-C30; $P < 1 \times 10^{-6}$). Participants that did not achieve complete transfusion independence during eculizumab treatment also experienced significant improvement in fatigue and other measures of quality of life. Of the 87 participants, 4 in the eculizumab group and 9 in the placebo group had serious adverse events, none of which were considered to be treatment-related; all these patients recovered without sequelae. The most common adverse events reported in the eculizumab group were headache, nasopharyngitis, back pain and nausea. Headache and back pain occurred more frequently in the eculizumab group than in the placebo group. Anti-eculizumab antibody responses were of low titer, transient, and occurred at a frequency similar to that of placebo. The TRIUMPH study demonstrated that eculizumab is a well-tolerated and effective therapy for PNH.

After initiation of the TRIUMPH study, eculizumab was evaluated in SHEPHERD, an open-label, non-placebo controlled, phase 3 clinical safety and efficacy study in a more diverse PNH population including patients with marked thrombocytopenia and minimal transfusion requirements. Participants were first enrolled and treated in 2005, and eculizumab was administered for a total of 52 weeks using a dosing schedule identical to that of TRIUMPH. Ninety-seven individuals at 33 international sites were enrolled. Adverse events were similar to those with placebo in the TRIUMPH study. Hemolysis was significantly reduced leading to improved anemia with a reduction in or elimination of transfusion requirement, lessened fatigue and improved overall health-related quality of life[75]. Compared to the overall population, efficacy with eculizumab was similar in individuals who would be expected to have less severe disease, including patient subgroups with lower baseline hemolysis, mild anemia, and minimal or no pretreatment transfusion requirement[76].

Of the 195 individuals entering the pilot, TRIUMPH or SHEPHERD studies, 187 participants completed these studies and elected to receive eculizumab in a common 104-week phase 3b open-label extension study. Overall, there was no evidence of an increased incidence of infection across PNH studies with eculizumab as compared to placebo, including serious infections, severe infections or multiple infections. Two individuals vaccinated against *N. meningitidis* experienced meningococcal sepsis; they were treated and recovered without clinical sequelae. Serious hemolysis was not observed in any of the initial 16 participants who discontinued treatment. Long-term eculizumab treatment was associated with a reduction in hemolysis up to more than 54 months of continuous treatment. Importantly, the design of the phase 3b extension study specified that the thrombosis rate for each participant would be compared before and during eculizumab treatment. Early evaluation shows that the 7.37 thrombotic events per 100 patient-years experienced before eculizumab was reduced to 1.07 events per 100 patient-years with eculizumab treatment ($P < 1 \times 10^{-6}$)[77].

Approval of eculizumab. After completion of TRIUMPH and after the pre-specified interim 26-week analysis in SHEPHERD, applications for marketing authorization of eculizumab in the treatment of PNH were submitted to the FDA and EMEA in September 2006. The FDA

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accepted the application for priority review and in March 2007, less than 6 months after the completion of SHEPHERD, FDA approved eculizumab for the treatment of patients with PNH to reduce hemolysis. The EMEA granted review of the eculizumab application under the Accelerated Assessment procedure, the fastest evaluation time frame for full approval awarded by EMEA. In June 2007, eculizumab became the first medicinal product to receive approval by the European Commission within the Accelerated Assessment procedure and it is indicated for the treatment of patients with PNH.

Conclusions and perspectives

Clinical development of eculizumab spanned a full decade before its first approval in the rare, disabling and life-threatening, clonal blood disorder PNH (**Fig. 5**). The studies before initiation of the PNH clinical program provided an important assessment of the safety profile and immunomodulatory effects of eculizumab. Once initiated, the PNH program has been one of the most extensive pre-approval clinical programs for an 'ultra-orphan' disease; the design of the multinational trials enrolling 195 participants allowed sequential, controlled and long-term evaluation of both biochemical and clinically validated measurements in the PNH population. This paradigm of careful evaluation may be used in the consideration of eculizumab in other uncommon, debilitating and life-threatening diseases for which there is evidence suggesting that terminal complement may significantly contribute to the pathophysiology of the disease, including antibody-mediated transplant rejection[78], myasthenia gravis and other peripheral neuropathies[79–83], fulminant lupus[27,29], catastrophic anti-phospholipid syndrome[84,85] and pattern 2 multiple sclerosis[86]. The understanding of critical pharmacokinetic and pharmacodynamic properties in PNH patients and the accomplishment of large-scale commercial manufacturing of eculizumab may also permit the evaluation of new formulations of eculizumab in future development programs for still other diseases with evidence suggesting complement-mediated pathology, including asthma[87] and age-related macular degeneration[88].

Clinical trials have demonstrated that eculizumab dramatically and reproducibly abrogates intravascular hemolysis, the primary clinical manifestation of PNH. These trials have shown that the reduction in hemolysis is independently associated with improvements in major morbidities in individuals with PNH including anemia, fatigue and health-related quality of life, as well as fewer thrombotic events. Because of the extremely rare nature of the disease and the ethical dilemma posed by the efficacy evident with eculizumab in the treatment of PNH, a prospective randomized controlled clinical trial is not a feasible means to examine the long-term impact of eculizumab on survival in individuals with PNH. However, given the evident anti-hemolytic effect and attendant clinical benefit of eculizumab administration, it is possible that eculizumab could beneficially impact several of the leading causes of premature mortality in PNH including venous and arterial thrombosis, hemorrhage that may be worsened by platelet consumption, renal failure and liver failure. In a *post hoc* analysis of the survival of eculizumab-treated PNH patients compared to historical controls[64], ~15 deaths would have been expected during the 2-year period of eculizumab treatment; however, two deaths were observed during this period, or 87% fewer than expected ($P = 0.0005$). This preliminary analysis suggests that long-term eculizumab treatment may beneficially affect survival in individuals diagnosed with PNH.

The humanization strategy used to construct eculizumab has resulted in only very rare evidence of responses to eculizumab, with an incidence similar to that found with placebo treatment. There is no evidence of tolerance or a neutralizing antibody response in any treated individual to date and infusion-related immune effects are similar in frequency to placebo. It is interesting to speculate that the selection of purely germline framework acceptor regions, seen as native by all recipients, has allowed for this important safety benefit.

Rigorous evaluation of eculizumab in PNH has helped tease out the role of hemolysis in mediating critical patient-reported outcomes, including fatigue and patient functioning. The reproducible demonstration that patient improvement with eculizumab, due to the abrogation

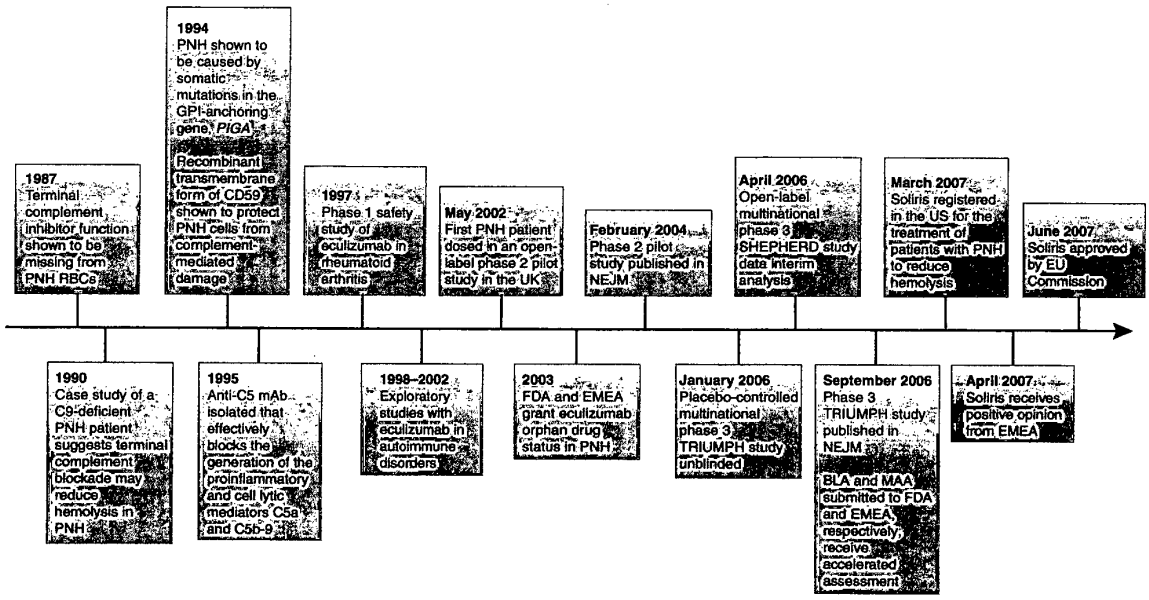


Figure 5 Eculizumab in PNH development timeline. BLA, biologic license application; MAA, marketing authorization application.

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of the underlying hemolysis, is distinct from associated improvements in anemia may have applications to understanding the potential multifactorial etiology of fatigue in individuals with anemia in other disease settings, such as cancer. Similarly, evident improvement in smooth muscle dystonias and thrombosis with eculizumab treatment in PNH has helped to establish new and important clinical paradigms, particularly with regard to the role of cell-free plasma hemoglobin and nitric oxide scavenging in thrombosis, smooth muscle dystonia and vasomotor abnormalities[53]. In this case, focus on a rare disease may not only lead to benefit for afflicted individuals but may also contribute to the overall foundation of knowledge. Such knowledge may provide insight into more common diseases with disordered nitric oxide regulation, including systemic and pulmonary hypertension, diabetes, sickle cell disease, and other genetic or acquired hemolytic anemias.

In summary, data from six clinical studies, enrolling a total of 195 participants, demonstrate the robust efficacy of eculizumab for the treatment of individuals with PNH. Every individual treated with eculizumab to date has shown an objective improvement in hemolysis. Treatment with eculizumab modifies the clinical course of PNH and markedly reduces the serious morbidities associated with the disease. In particular, therapy with eculizumab reduces anemia, blood transfusion requirements, and the risk for life-threatening thrombosis. Eculizumab also improves other morbidities that are often serious, including fatigue, pain, dyspnea and impaired overall quality of life. Treated individuals will continue to be monitored in a global registry. Overall, the therapeutic benefit and safety of eculizumab in the treatment of patients with the rare blood disease PNH validates the utility of terminal complement inhibitor therapeutics for the treatment of medical conditions where complement activation is involved.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturebiotechnology/.

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Erratum: Funding crunch forces stem cell company to abandon therapies

Monya Baker
Nat. Biotechnol. 25, 951–952, 2007; published online 3 September 2007

In the version of this article originally published, in the penultimate paragraph, the name of the Monash University scientist was misspelled; the correct spelling is Colin Pouton. In addition, the statement in paragraph 2 "the SSCC, which is being supported by the same group that funded ESI, the Agency for Science, Technology and Research (A*STAR)" is incorrect. ESI is not funded by A*STAR. Its major investor is Bio-One Capital, a venture capital fund controlled through the Singapore Government's Economic Development Board. The SSCC is funded by A*STAR.

Erratum: Mice with a human touch

Christopher Thomas Scott
Nat. Biotechnol. 25, 1075–1077, 2007; published online 6 October 2007

In the version of this article initially published, it was incorrectly stated on p. 1076, paragraph 2, line four from bottom, that "Aya Jakobovits, originally at Cell Genesys and later at Abgenix, shepherded the XenoMouse through its entire development." Five of the six XenoMouse strains developed at Abgenix were initiated after Jakobovits left in 1997.

Erratum: Discovery and development of the complement inhibitor eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria

Russell P Rother, Scott A Rollins, Christopher F Mojcik, Robert A Brodsky & Leonard Bell
Nat. Biotechnol. 25, 1256–1264 (2007); published online 7 November 2007; corrected after print 7 December 2007

In the version of this article initially published, on p. 1258, paragraph 2, the incorrect serum concentrations to reach steady state and the incorrect accumulation ratio (R_{acc}) for eculizumab were given. The corrected sentence reads "Eculizumab serum concentrations appear to reach steady state after ~57 days. At steady state, the eculizumab accumulation ratio (R_{acc}) was calculated to be 1.075." In addition, in Figure 5, in the PDF version only, the word "assessment" was cut off the bottom of the box beginning "September 2006." The final sentence should read "BLA and MAA submitted to FDA and EMEA, respectively; receive accelerated assessment." And in Table 1, the heading "Units of packed RBCs transfused/patient/month" now appears in a separate row. The errors have been corrected in the HTML and PDF versions of the article.

Corrigendum: A diverse family of thermostable cytochrome P450s created by recombination of stabilizing fragments

Yougen Li, D Allan Drummond, Andrew M Sawayama, Christopher D Snow, Jesse D Bloom & Frances H Arnold
Nat. Biotechnol. 25, 1051–1056 (2007); published online 26 August 2007; corrected after print 7 December 2007

In the version of this article initially published, the sentence starting with "The most thermostable 450 P450 chimera" contains an extra "450." The correct sentence should start as follows "The most thermostable P450 chimera". The error has been corrected in the HTML and PDF versions of the article.

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Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
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Blood concentrations of alemtuzumab and antiglobulin responses in patients with chronic lymphocytic leukemia following intravenous or subcutaneous routes of administration

Geoff Hale, Peppy Rebello, Lee R. Brettman, Chris Fegan, Ben Kennedy, Eva Kimby, Mike Leach, Jeanette Lundin, Håkan Mellstedt, Paul Moreton, Andy C. Rawstron, Herman Waldmann, Anders Osterborg and Peter Hillmen

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Blood concentrations of alemtuzumab and antiglobulin responses in patients with chronic lymphocytic leukemia following intravenous or subcutaneous routes of administration

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Alemtuzumab is a humanized anti-CD52 antibody licensed for refractory B-cell chronic lymphocytic leukemia (B-CLL), when given intravenously at 30 mg thrice weekly. However, the intravenous route is associated with infusion-related reactions and is inconvenient. We measured blood concentrations in 30 relapsed patients treated with intravenous alemtuzumab and in 20 patients from a previously untreated group who received similar doses subcutaneously. Highest trough samples in the intravenous group were less than 0.5 μg/mL to 18.3 μg/mL (mean 5.4 μg/mL). The cumulative dose required to reach 1.0 μg/mL was 13 mg to 316 mg (mean 90 mg). Higher blood concentrations correlated with the achievement of better clinical responses and minimal residual disease. The highest measured concentrations in the subcutaneous group were similar (0.6 μg/mL to 24.8 μg/mL, mean 5.4 μg/mL). However, the cumulative dose to reach 1.0 μg/mL was higher: 146 mg to 1106 mg (mean 551 mg). No antiglobulin responses were detected in 30 patients given intravenous alemtuzumab whereas 2 of 32 patients given subcutaneous alemtuzumab made substantial anti-idiotype responses. Thus, subcutaneous alemtuzumab achieved concentrations similar to those for intravenous alemtuzumab, although with slightly higher cumulative doses. Subcutaneous alemtuzumab is more convenient and better tolerated but may be associated with some patients forming anti–alemtuzumab antibodies, particularly those patients who were previously untreated. (Blood. 2004;104:948-955)

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Introduction

Alemtuzumab (CAMPATH-1H, Campath; ILEX Pharmaceuticals, San Antonio, TX) is a humanized immunoglobulin G1 (IgG1) antibody that recognizes the CD52 antigen, a lipid-anchored glycoprotein, on lymphocytes.[1-3] Alemtuzumab is exceptionally lympholytic and has been tested as an immunosuppressive agent in transplantation and autoimmune diseases. It is active against a range of lymphoid malignancies and in 2001 was approved for the treatment of B-cell chronic lymphocytic leukemia (B-CLL) in patients who have been treated with alkylating agents and who failed fludarabine therapy. This was based largely on a trial in 93 patients, most of whom had advanced disease with an extremely poor prognosis.[4] After a short dose escalation, alemtuzumab was given intravenously at 30 mg thrice weekly for 4 to 12 weeks. This gave an overall response rate (complete remission [CR] + partial remission [PR]) of 33%. Other trials in chemoresistant CLL reported comparable response rates of 42% and 29% respectively.[5,6]

The dose regimen was developed empirically without the benefit of detailed pharmacodynamic or pharmacokinetic studies. Alemtuzumab often causes substantial first-dose reactions, and although the reactions can now be minimized by appropriate premedication, they are the reason why an initially escalating dose was followed by frequent small doses. This regimen is not the most convenient for patients or physicians. Therefore, the subcutaneous route has been studied in an attempt to reduce side effects and make the treatment more manageable.[7,8]

The different routes of administration can be compared by analysis of the biodistribution of antibody, on the principle that treatments that give comparable blood concentrations should produce similar clinical effects. Some information is available about the pharmacokinetics of intravenous alemtuzumab in patients undergoing stem cell transplantation who have no substantial leukemic burden.[9,10] The terminal half-life was estimated to be approximately 15 to 20 days. However, in patients with advanced CLL, tumor cells could absorb antibody from the circulation.

The humanization of alemtuzumab reduced the risk of antiglobulin responses.[11] However, the antigen binding sites are still potentially immunogenic. The ability to provoke a response depends on the target antigen, the immune status of the patient, the use of immunosuppressive drugs, and other factors, perhaps including the route of administration. There is a suggestion that subcutaneous administration might be more immunogenic than

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intravenous administration in patients with rheumatoid arthritis,[12,13] though it is well known that such patients are particularly able to make anti–Ig antibodies.

Here we measured blood concentrations of alemtuzumab and antiglobulin responses against it during 2 different clinical trials. In the first, alemtuzumab was administered intravenously to patients who had previously failed alkylating agents and fludarabine as in the licensed indication. In the second, alemtuzumab was administered subcutaneously to patients who required therapy but had received no prior treatment.[8] We compared the blood concentrations and antiglobulin responses and investigated the possible relationship between alemtuzumab concentrations and clinical responses.

Patients, materials, and methods

Patients

Clinical studies were carried out with the approval of local and, in the United Kingdom, regional ethical committees. All patients gave written informed consent prior to enrolment.

Study design: intravenous study

An open-label phase 2 trial was carried out at 5 centers in the United Kingdom (Aberdeen, Birmingham, Bournemouth, Glasgow, Leeds). Primary objectives included the assessment of safety and efficacy as well as measurement of pharmacokinetics. Patients were eligible if they were at least 18 years old, had confirmed B-CLL requiring treatment, had failed previous treatment with purine analogs, had a World Health Organization (WHO) performance status of 2 or less, and had creatinine and bilirubin concentrations less than twice the upper limit of normal (unless due to disease). Exclusion criteria were active infection, HIV positivity, pregnancy or lactation, history of anaphylaxis to antibodies, less than 3 weeks since chemotherapy or less than 6 weeks since investigational therapy, central nervous system (CNS) involvement or persisting severe pancytopenia, other severe concurrent diseases, secondary malignancy, or mental disorders. Patients were premedicated with paracetamol and chlorpheniramine 30 minutes before the first infusion. Septra (sulfamethoxazole/trimethoprim 800 mg/160 mg twice a day, 3 times per week) and aciclovir (200 mg thrice daily) were given prior to therapy and continued until normal blood lymphocytes returned to the normal range. Allopurinol was given during the first 4 weeks of treatment. Alemtuzumab (M&I Partnership, Cambridge, MA) was diluted in 100 mL of 0.9% saline and administered intravenously over approximately 2 hours. A dose of 3 mg was given on day 1 and if it was well tolerated, a 10-mg dose was given on day 2 and a 30-mg dose on day 3. If severe reactions occurred, the same dose was repeated daily until it was well tolerated and then the dose was escalated. Subsequent 30-mg doses were given thrice weekly for up to 12 weeks, though treatment was occasionally extended if clinically indicated.

Before therapy, a full history, physical examination, and laboratory evaluation were performed, including assessment of lymph node, liver, and spleen size, computed tomographic (CT) scan, Rai staging, blood analysis, bone marrow aspirate, and trephine and immunophenotyping by flow cytometry. Disease parameters were reassessed and blood and bone marrow were reanalyzed by flow cytometry for residual CLL cells every 4 weeks. Therapy was stopped if there was no evidence of CLL or if sequential bone marrow samples showed no fall in the level of CLL. Blood samples were collected weekly to measure alemtuzumab concentrations and antiglobulins. After the last dose, samples were collected daily for 4 days and then weekly for 8 weeks. This was not always possible for logistic or clinical reasons.

During follow-up, patients were assessed monthly for 6 months. Responders were assessed every 6 months thereafter until disease progression. Disease response and toxicity were graded according to the criteria of the National Cancer Institute (NCI).

Study design: subcutaneous study

An open-label phase 2 trial was conducted at 4 clinics at the Karolinska Institute, Stockholm, Sweden.[8] The primary objective was to assess the efficacy of alemtuzumab administered subcutaneously. Patients were eligible if they had a diagnosis of B-CLL, were age 18 to 75, had a WHO performance status of 1 or less, life expectancy at least 12 weeks, required treatment, and had not been treated previously. Other details were comparable to the intravenous study except the treatment regimen. Alemtuzumab was obtained from the Therapeutic Antibody Centre, Oxford, United Kingdom (patients 1-29) or from M&I Partner (patients 30-41). The 2 preparations were similar although the formulations were slightly different and antibody from the Therapeutic Antibody Centre was stored frozen.[14] On day 1, 3 mg alemtuzumab was administered by subcutaneous injection in the thigh. If well tolerated, 10 mg was given on day 3 and 30 mg (in 2 injection sites) was given on day 5. In the event of erythema or edema, the same dose was repeated until well tolerated. After dose escalation, most patients self-administered the antibody. The 30-mg dose was given thrice weekly for up to 18 weeks. If treatment was interrupted for more than 7 days the dose was reinitiated at 3 mg or 10 mg. Therapy was stopped if patients achieved a complete remission (CR) or fulfilled NCI criteria for progressive disease (PD).

Measurement of alemtuzumab in patient serum

Serum samples were collected before doses and at various times afterward and stored at −70°C until analysis by immunofluorescence.[15] Test samples were incubated at 56°C for 30 minutes to inactivate complement then incubated with HUT-78 cells at room temperature for 30 minutes. The cells were washed and resuspended in fluorescein isothiocyanate (FITC)–labeled polyclonal anti–human IgG Fc domain (F-9512; Sigma, Poole, United Kingdom), incubated for 30 minutes, washed and fixed with formaldehyde prior to flow cytometry. Alemtuzumab concentrations were calculated from the median fluorescence by interpolation on a standard curve. The lower limit of quantitation was 0.5 μg/mL (allowing for 2-fold sample dilution), overall precision was plus or minus 13%, and overall accuracy was 109%. There was no interference by normal or patient control sera and no reactivity with $F(ab')_2$ fragments of alemtuzumab.[15]

Measurement of antiglobulin responses

Antiglobulin responses to alemtuzumab were measured in serum samples by sandwich enzyme-linked immunosorbent assay (ELISA) as previously described[11,16] with some modifications. Microtiter plates were coated with alemtuzumab (M&I Partner) and blocked with diluent (phosphate-buffered saline [PBS] containing 2% bovine serum albumin [BSA]). Standards, quality-control samples, and test samples were added, incubated for 1 hour at room temperature, and rinsed 4 times with wash buffer (PBS containing 0.05% Tween 20). Biotin-labeled alemtuzumab was added to each well, incubated for 1 hour, and rinsed as before. The assay was developed with extravidin-peroxidase (E-2886; Sigma) and ortho-phenylenediamine. Color development was measured at 450 nm for 20 minutes by Genesis II software (Thermo Life Sciences, Basingstoke, United Kingdom). The standard was YID13, a monoclonal anti-idiotype specific for alemtuzumab.[16] One U/mL of antiglobulin activity is the signal equivalent to 1 ng/mL of YID13 reference standard. The limit of detection (LOD) and lower limit of quantitation (LLOQ) was 488 U/mL serum (allowing for 2-fold sample dilution), overall precision was plus or minus 14%, and overall accuracy was 94%. There were no false positives and no interference by normal or patient control sera. Positive signals were obtained with serum containing rheumatoid factor or spiked with native CD52 antigen. The assay was reproducible despite variations in reagent concentrations, incubation and washing conditions, blood clotting conditions, and sample storage conditions within the specified ranges. Up to 3-fold enhancement of antiglobulin activity was seen in samples that had been heat-treated or stored for over a month at 4°C. This might be due to aggregation, since aggregates of the control anti-idiotype reagent also gave enhanced activity. Alemtuzumab and anti-alemtuzumab interact with each other to reduce the signal in either assay if both were present in the same sample. Thus, the

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antiglobulin assay measures only the concentration of free anti–alemtuzumab antibody, that is, the excess over the levels of alemtuzumab itself. Considering that alemtuzumab concentration was never more than 26 μg/mL, then it might interfere with measurement of antiglobulin responses less than about 26 000 U/mL. However, the antiglobulin levels in a patient who made a clear-cut response exceeded 10^6 U/mL. We report data for all samples measured, both during and, where available, after treatment. For complete assessment of antiglobulin responses, it is always preferable to analyze samples over an appropriate period of time after the end of treatment.

Measurement of minimal residual disease by flow cytometry

Residual CLL cells were quantified by sensitive 4-color flow cytometry; a method more sensitive than consensus-primer polymerase chain reaction (PCR) and more practicable than sequence-specific quantitative PCR.[17] At least 5×10^6 bone marrow leukocytes were incubated with a mixture of fluorescent-labeled antibodies, washed twice, and analyzed using a FACSort (Becton Dickinson, Oxford, United Kingdom) with CELLQuest v3.1 software. The antibodies used were CD45/CD48 (Chemicon, Chandlers Ford, United Kingdom)/CD19/CD3, kappa/lambda/CD19/CD5, CD20 (Beckman Coulter, High Wycombe, United Kingdom)/CD79b (Beckman Coulter)/CD19/CD5, and CD20 (Beckman Coulter)/CD38 (BD Biosciences, Oxford, United Kingdom)/CD19/CD5 conjugated to FITC/phycoerythrin (PE)/PE-Cy5/allophycocyanin (APC), respectively. Reagents without named manufacturer were produced in-house. Between 50 000 and 500 000 cells were analyzed. B cells were identified by setting a region on CD19 versus side scatter (SSC), followed by a forward scatter region, and then ensuring that no $CD3^+$ events fell within the combined gate. CLL cells were discriminated from mature B cells by their stronger expression of CD5 and weaker expression of CD20 and CD79b, and from B progenitors by their weaker expression of CD38. This assay can detect 1 CLL cell in approximately 10^4 to 10^5 leukocytes.

Results

Collection and analysis of samples

Serum samples were obtained from all 30 patients in the intravenous study and 20 of the 41 patients in the subcutaneous study, almost exclusively from a single clinic (Karolinska Hospital). Samples were tested in duplicate and the mean concentration was calculated. If the mean was less than 0.5 μg/mL (LLOQ), a level of less than 0.5 μg/mL was recorded. If either result was more than 20 μg/mL or a measurement was technically faulty, then the test was repeated, using a higher dilution if appropriate, and the repeat results were used.

Optimal time for collection of peak samples following intravenous administration

A preliminary experiment was carried out to identify the time following intravenous administration when the peak antibody concentration might be found. Samples were collected from 3 patients at 15 minutes, 30 minutes, and 60 minutes after the end of one dose each week for 4 to 7 weeks. The peak sample occurred at 15 minutes, significantly more often (21 times) than at 30 minutes (6 times) or 60 minutes (2 times) ($P < .01$; paired Wilcoxon signed rank test). Therefore, for subsequent patients a single sample was collected at 15 minutes to determine the peak concentration.

Serum concentrations of alemtuzumab during intravenous treatment

A total of 1561 patient samples were tested. Terminal samples (after the last dose) were not available for some patients, and in a few cases only a small number of terminal samples were available.

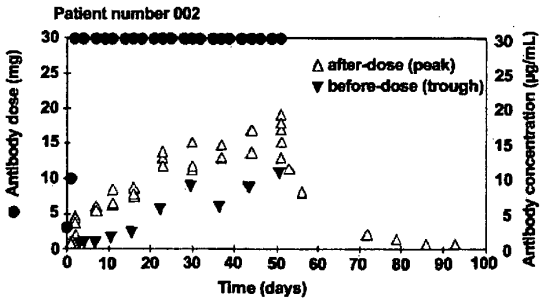


Figure 1. Example of alemtuzumab concentrations in patient treated with intravenous antibody. After an initial dose escalation, 30 mg of alemtuzumab was administered 3 times a week for 8 weeks (●). Serum samples were taken before (Δ) and after (▼) the dose once a week. This patient made a good clinical response to the therapy.

All samples were tested in duplicate; the overall mean coefficient of variation (CV) was 10%. Many of the early samples and many trough samples were below the limit of quantitation (< 0.5 μg/mL). Five samples gave anomalous results, substantially diverging from other results for the same patients. These could be explained by accidental mix-ups at the clinical centers and were omitted from analysis. An example of the doses and blood concentrations throughout the course of treatment for one patient is shown in Figure 1.

In contrast to patients who had received transplants,[9,10] there were wide variations in alemtuzumab concentrations between different patients with CLL. The highest peak ranged from 2.8 μg/mL to 26.4 μg/mL (mean 10.7 μg/mL). The highest trough ranged from less than 0.5 μg/mL to 18.3 μg/mL (mean 5.4 μg/mL). The cumulative dose before the trough concentration reached 1.0 μg/mL ranged from 13 mg to 316 mg (mean 90 mg; Table 1). In 9 of 21 evaluable patients, the highest trough concentration was measured just before the last dose, indicating that a steady state had not been reached. Total cumulative doses varied between patients, but did not correlate with the final antibody concentrations (Table 1).

Comparison of the starting lymphocyte count with the peak concentration following the first 30-mg infusion showed a modest negative correlation, that is, the higher the initial lymphocyte count, the lower tended to be the peak concentration of alemtuzumab (Figure 2).

In Figure 3 the highest trough concentration for each patient is plotted against the response measured by NCI criteria for 27 patients where the clinical response was reported within 4 months of the end of treatment. There was a significant increase in trough

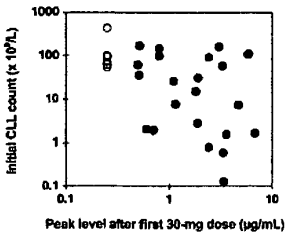


Figure 2. Relationship between initial CLL count and alemtuzumab blood concentration: intravenous study. The numbers of CLL cells in the blood directly before intravenous therapy are plotted against the peak concentration of alemtuzumab measured directly after the first 30-mg dose (●). In 8 patients the concentration was below the limit of quantitation, and these have all been plotted at an arbitrary level of 0.25 μg/mL (○). Pearson correlation coefficient for log (initial CLL count) versus log (alemtuzumab concentration) was −0.44, $P = .01$.

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Table 1. Alemtuzumab dose and serum concentrations: intravenous study

Patient no.	Cumulative dose, mg	Number of samples	Terminal samples	Dose to reach 1 μg/mL	Maximum peak, μg/mL	Maximum trough, μg/mL	Before last dose, μg/mL	After last dose, μg/mL
001	1026	80	12	143	16.0	5.5	7.7	11.7
002	873	66	12	13	19.1	13.0	13.0	19.1
003	1106	91	23	43	8.7	1.1	0.9	5.9
004	832	54	0	103	26.4	18.3	ND	ND
005	1054	88	7	46	16.3	6.2	6.2	16.3
006	736	55	8	16	9.5	3.7	2.6	5.5
007	1063	75	11	133	19.8	11.9	11.9	19.4
008	291	19	3	111	5.8	0.7	0.7	0.7
009	1026	73	12	63	13.1	7.7	7.7	12.2
010	179	21	0	46	4.0	0.8	ND	ND
011	1075	71	11	133	8.3	1.9	1.9	8.3
012	473	28	0	53	4.4	0.2	ND	ND
013	946	60	13	163	4.0	0.5	< 0.5	2.9
014	436	31	0	46	12.5	9.9	ND	ND
015	2063	89	0	43	8.4	2.8	ND	ND
016	1016	56	11	103	9.5	4.9	4.5	9.0
017	1093	55	8	133	8.6	1.7	1.2	6.0
018	733	40	1	43	8.2	2.6	ND	ND
019	463	27	1	73	19.9	14.8	ND	ND
020	1093	56	12	43	17.7	9.4	7.7	12.9
021	583	32	0	163	4.0	1.1	ND	ND
022	1003	46	9	43	7.1	1.0	0.8	6.3
023	1063	47	6	43	29.0	17.0	ND	24.1
024	1126	67	12	316	8.0	2.6	2.6	5.5
025	1063	64	11	73	6.9	5.2	5.1	6.7
026	968	57	13	133	5.4	1.5	1.4	4.7
027	1066	45	10	73	2.8	0.9	< 0.5	2.5
028	673	42	13	43	8.5	4.0	4.0	6.9
029	416	40	10	133	7.6	3.3	3.3	7.6
030	763	35	5	133	12.2	3.7	3.7	12.2
Minimum	179	19	0	13	2.8	< 0.5	< 0.5	0.7
Mean	873	52	8	90.4	10.7	5.4	4.0	9.4
Maximum	2063	91	23	316	26.4	18.3	13.0	24.1

ND indicates not determined.

concentrations with increasing clinical response ($P = .006$, Kruskal-Wallis test). There was an even more striking correlation between trough concentrations and the attainment of minimal residual disease (MRD) negativity in 7 patients (filled symbols) compared with the 17 patients who still had residual CLL cells in the bone marrow at the end of treatment as measured by 4-color flow cytometry ($P < .0001$, Kruskal-Wallis test)

In Figure 4 the mean trough concentration is plotted throughout the treatment for 2 groups of patients: (1) 8 responders who reached MRD less than 0.4%, and (2) 22 others. The mean trough concentration was significantly higher in the responders throughout the whole course of treatment from week 2 to week 11 ($P < .02$ at each time point, Kruskal-Wallis test).

Clearance of alemtuzumab following the last dose showed a similar correlation with residual disease. In patients with undetectable CLL cells, there was a single clearance phase characterized by

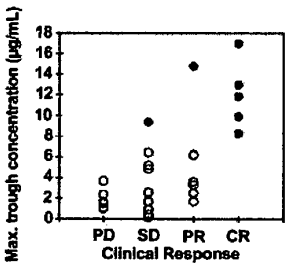


Figure 3. Relationship between maximum trough concentrations of alemtuzumab and clinical response: intravenous study. The highest trough concentration of alemtuzumab (which generally occurred following the penultimate or last dose) is plotted against the clinical response at the end of intravenous treatment as determined by NCI criteria. PD indicates progressive disease; SD, stable disease; PR, partial remission; CR, complete remission. A better clinical outcome was significantly associated with higher alemtuzumab concentrations ($P = .006$, Kruskal-Wallis test). There was an even stronger correlation between high trough concentrations (> 8 μg/mL) and good responses measured by less than 0.1% CLL cells in the bone marrow (●; $P < .0001$). Three patients who died before the end of the planned treatment course are not included in this figure. Of those patients, 2 had low alemtuzumab concentrations (≤ 1.1 μg/mL) and progressive disease at the time of death. The third had a high antibody concentration (18.3 μg/mL) and low levels of residual disease (0.21%). Each symbol represents a single patient; ● indicates a patient with no detectable MRD; ○, a patient with detectable MRD.

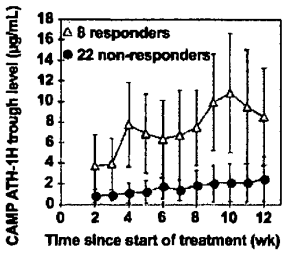


Figure 4. Mean trough concentrations of alemtuzumab during intravenous therapy. The mean trough concentrations during treatment (measured 48 hours after a dose, once a week) are plotted, with standard deviations, for 8 patients who ultimately reached less than 0.4% CLL cells in the bone marrow (△) and compared with 22 patients who still had residual CLL cells at the end of treatment (●).

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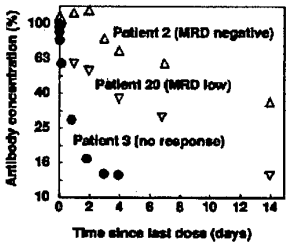


Figure 5. Clearance of alemtuzumab in selected patients treated intravenously. In patients with minimal residual disease (by flow cytometry) at the end of therapy, clearance was slow and single phase (△). Patients who still had a small amount of CLL cells showed biphasic clearance (▽) and patients who still had bulky disease cleared the antibody rapidly (●). However, in each case the terminal half-life was roughly similar.

a relatively long half-life. In patients who still had a substantial tumor burden, most of the antibody was rapidly cleared from the blood. Patients with low levels of residual disease showed an intermediate pattern. An example of each is shown in Figure 5.

Sixteen patients contributed informative terminal samples after the last 30-mg dose. From these data the following mean pharmacokinetic parameters were calculated (ILEX Oncology, unpublished data, September 2001). The apparent steady-state volume of distribution (V_{SS}) was 0.185 L/kg and the apparent volume of distribution during the terminal phase (VZ) was 0.252 L/kg. These are larger than for other monoclonal antibodies in humans but are consistent with the notion that alemtuzumab distributes through the plasma compartment and a substantial extravascular lymphocyte compartment. The mean terminal phase half-life ($t_{1/2,\ z}$) was 6.1 days, which is consistent with previous measurements for alemtuzumab and other chimeric antibodies in humans.

Serum concentrations of alemtuzumab during subcutaneous treatment

This study was started in 1998 using alemtuzumab manufactured at the Therapeutic Antibody Centre. During June 2000, commercially produced material became available and was subsequently used. No obvious differences were observed in the clinical responses. A total of 542 patient samples were received and tested. Two were mislabeled and are not included here. The overall mean CV was 8%. Assessment of pharmacokinetics in 7 patients was inadequate due to the limited number or inappropriate timing of samples.

Patient no. 8036 (who started with alemtuzumab from the Therapeutic Antibody Centre and was switched to commercial alemtuzumab) never achieved concentrations above the LLOQ. This patient had rapidly made a strong antiglobulin response to the alemtuzumab, which probably neutralized all of the administered antibody ("Antiglobulins following subcutaneous treatment"). The patient never showed any clearance of lymphocytes but showed continued reactions at the injection site. Because of this, the patient was switched to intravenous administration after 6 weeks.

There were some changes from the dose regimen due to clinical reactions. There were 2 patients who received lower doses throughout (10 mg and 20 mg) and 2 (including patient no. 8036, above) who were switched to intravenous dosing. The dose escalation rate was often slower than originally planned, typically taking 1 to 2 weeks to reach 30 mg. After this, the planned dose was usually maintained but only 1 of the 21 patients analyzed received the maximum planned dose of 1573 mg. The mean total dose was 1249 mg and the lowest dose was 712 mg.

The highest concentrations ranged from 0.6 μg/mL to 24.8 μg/mL and the mean (5.4 μg/mL) was the same as in the intravenous study (Table 2). However, the cumulative dose before the concentration reached 1.0 μg/mL ranged from 146 mg to 1106 mg (mean 551 mg), which was substantially more than in the intravenous study. An example of the time course in one patient illustrating this delay is shown in Figure 6.

Inadequate samples were available for analysis of terminal phase pharmacokinetics. No correlation could be demonstrated between alemtuzumab concentrations and clinical responses because nearly all of the patients (18/20) were responders (CR or PR), and levels of MRD were not reported.

Antiglobulins following intravenous treatment

A total of 519 serum samples from 30 patients treated with intravenous alemtuzumab were tested for antiglobulins. These included all samples taken more than 24 hours after the last dose and representative pre-dose samples during treatment. All of them were below the limit of detection (488 U/mL). In 12 patients there were no or very few samples available after the end of treatment, and in the other 18 patients samples were only available between 2 and 6 weeks after the last dose. Therefore, we cannot completely exclude the possibility of delayed responses.

Antiglobulins following subcutaneous treatment

A total of 281 samples were tested from 21 patients (one patient had adequate late samples for antiglobulin measurement, but none suitable for pharmacokinetic analysis). These included all samples taken after the last dose and representative samples taken during treatment. There were 4 patients who had no or few samples after the end of treatment but 17 who had adequate posttreatment samples. All samples except those described below were below the LOD.

One patient (no. 8027) had a single sample taken at day 238 (114 days after the last dose) that gave a weak signal just above the LOD. All previous and subsequent samples were less than the LOD. The patient made a good clinical response to therapy, achieving a CR, and no relevant adverse effects were noted. It is possible that the patient made a very weak, transient response to the alemtuzumab, but we doubt that it could have been clinically significant.

One patient (no. 8036) made a very high-titer response, reaching a peak of approximately 3.7×10^6 U/mL 24 days after the last dose of alemtuzumab (Figure 7). This antiglobulin bound strongly to CAMPATH-1G, the parental rat antibody from which alemtuzumab was humanized, but did not bind other human IgG1

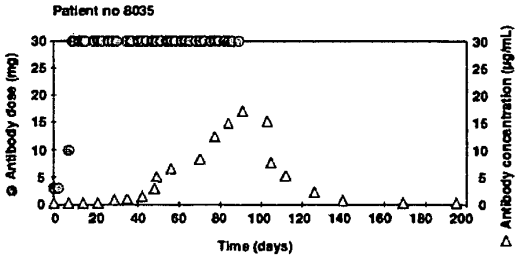


Figure 6. Example of alemtuzumab concentrations in a patient treated with subcutaneous antibody. After an initial dose escalation, 30 mg of alemtuzumab was administered 3 times a week for 12 weeks (○). Serum samples were taken before the dose, once a week (△). This patient made a good clinical response to the therapy and achieved relatively high concentrations of antibody.

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Table 2. Alemtuzumab dose and serum concentrations: subcutaneous study

Patient no.	Cumulative dose, mg	No. of samples	Above LOQ	Terminal samples	Dose to reach 1 μg/mL	Max concentration, μg/mL	After last dose, μg/mL
8016	1368	24	2	0	729	1.3	ND
8015	1506	28	1	0	> 156	0.8	ND
8018	1573	29	3	0	673	1.5	ND
8017	1213	20	2	0	> 193	0.9	ND
8021	883	29	12	2	353	5.3	4.1
8026	1378	23	3	0	550	1.4	ND
8027	1269	43	9	1	630	6.6	6.6
8028	1363	37	15	3	403	5.8	5.8
8029	1133	34	8	1	603	7.3	7.3
8030	1553	45	16	1	473	4.3	4.2
8031	712	27	2	1	712	1.4	1.4
8032	723	17	2	0	393	2.5	2.5
8033	1502*	28	10	0	542	3.4	3.0
8034	1412	24	16	4	269	6.3	5.8
8035	1035	24	15	6	456	17.1	17.1
8036	819*	21	0	0	> 819	< 0.5	< 0.5
8038	1465	19	8	3	1108	2.6	2.6
8039	1526	19	10	0	506	4.4	ND
8040	1496	28	19	7	146	24.8	24.8
8041	852	23	6	0	462	3.5	3.5
Minimum	712	17	0	0	146	0.6	< 0.5
Mean	1249	27	8	1.6	551	5.4	6.4
Maximum	1573	45	19	7	1108	24.8	24.8

*Some doses for patients no. 8033 and no. 8036 were given intravenously due to infusion site reactions during the subcutaneous administration.

monoclonal antibodies. Therefore, essentially all of the response was directed against the alemtuzumab idiotype. No free alemtuzumab could be detected throughout treatment and there was no significant depletion of tumor cells during therapy. This patient developed a pronounced injection site reaction including erythema, edema, and slight local pain but otherwise no marked general symptoms. As a result, the patient did not achieve the 30-mg dose level during 6 weeks of subcutaneous dosing and alemtuzumab was subsequently administered intravenously. Due to nonresponsiveness, the patient was subsequently treated with fludarabine and went into a long-lasting partial remission. He was retested 18 months after the end of alemtuzumab treatment (continuing in PR) and still had a high-titer antiglobulin (approx 1.8×10^5 U/mL). There was a very low anti-alemtuzumab signal in one of 2 samples taken before therapy. This patient might have had a natural anti-IgG (rheumatoid factor) prior to therapy and perhaps was predisposed to make an antiglobulin response.

A search was made for samples from other patients who had received subcutaneous alemtuzumab. Samples were collected between 4 to 50 months after the end of treatment from an additional 9 patients of the original subcutaneous study plus 2 others who received subcutaneous alemtuzumab as part of a compassionate program. A substantial response was detected in patient no. 8020 of approximately 4800 U/mL at 32 months after the end of treatment. Like the response in patient no. 8036, this response cross-reacted on the parental rat antibody but not on other human antibodies, showing it to be essentially anti-idiotype. The patient had not responded to the therapy, but was reported to have prolonged skin reactions to the infusions. A very weak response was detected in one other patient of 541 U/mL, barely above the LOD. Unlike the other 2 patients, this serum cross-reacted with other human IgG but not with CAMPATH-1G. We consider that it is a nonspecific rheumatoid-like factor and probably had no clinical significance with respect to alemtuzumab.

In total therefore, of 32 patients treated with subcutaneous alemtuzumab (30 who were previously untreated with any anti-CLL agent), 2 produced a significant antiglobulin response which persisted at potentially neutralizing concentrations for more than a year. Both of these patients had received alemtuzumab manufactured at the Therapeutic Antibody Centre, as did most of the patients in this study, although patient no. 8036 had been switched to the commercial product after 3 weeks.

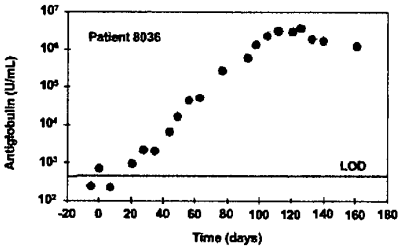


Figure 7. Antiglobulin response to alemtuzumab following subcutaneous treatment. This patient received subcutaneous alemtuzumab at doses up to 10 mg until day 37 (cumulative dose, 89 mg) and subsequently received 30 mg 3 times a week intravenously until day 80, followed by 30 mg 3 times a week subcutaneously until day 102. Samples were taken once a week to measure antiglobulin responses. One U/mL is equivalent to 1 ng/mL of a standard monoclonal anti-idiotype. The horizontal line indicates the limit of detection (LOD).

Discussion

Alemtuzumab is extensively used for patients with B-CLL who have failed treatment with alkylating agents and purine analogues and investigators are exploring its use in other disease settings. To enable safe and effective use it is essential to understand alemtuzumab's pharmacokinetics and biodistribution as well as the potential for anti-idiotype responses, which all may differ according to disease, patient status, and route of administration. We have started to address these issues by comparing blood concentrations of

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alemtuzumab and antiglobulin responses in 2 different studies for treatment of CLL, namely intravenous treatment of chemotherapy-resistant disease and first-line subcutaneous treatment. The studies were initiated independently and had different objectives; nevertheless the pharmacologic results are instructive.

Alemtuzumab may cause substantial "first dose" reactions as a result of cytokine release when it is given intravenously.[3,18,19] Therefore, the standard treatment regimen involves dose escalation and frequent, relatively small doses, though it has not been formally established whether the reactions are strictly dose related. A treatment course for chemotherapy-resistant CLL consists of approximately one gram of the antibody. However, a cumulative dose of 40 mg to 100 mg is highly immunosuppressive and clinically effective in patients without bulky tumors, for example those treated for prevention of transplant rejection,[11,20] multiple sclerosis,[21] and other autoimmune diseases,[22-24] as well as to eradicate detectable minimal residual disease in CLL.[25] The biodistribution of alemtuzumab is likely to depend on the bulk of tumor cells, possibly more so than other monoclonals because CD52 is such a highly expressed antigen.

There are approximately 5×10^5 binding sites for alemtuzumab on a normal lymphocyte.[26,27] There are about 10^{12} lymphocytes in a healthy adult.[28] At least 125 mg of alemtuzumab is therefore required to saturate all of the CD52 sites. CLL cells express a similar amount of CD52 antigen to normal lymphocytes[27] but their total number may be 10 times greater. Therefore, more than 1 g of alemtuzumab could be required to saturate all the receptors in some patients, especially considering that the antibody has a relatively low affinity and that there will be a significant clearance during a 12-week course of treatment. Hence, it is not surprising that the biodistribution and clearance of antibody during the treatment of patients with CLL is dominated by the tumor burden and cannot be fitted to a simple pharmacokinetic model.

There was a strong correlation between the concentrations of antibody throughout intravenous treatment and the ultimate clinical response, or the achievement of minimal residual disease (MRD) negativity in the bone marrow by flow cytometry. It has recently been reported that survival correlates better with MRD than with the standard NCI criteria for clinical responses.[29] Without a good measure for the initial total tumor burden it is difficult to distinguish cause and effect. One possibility is that patients with high tumor burden were treated with inadequate amounts of antibody to achieve sufficient receptor site saturation and see a clinical effect. In any event, it appears that measurements of alemtuzumab concentrations after 2 to 4 weeks of treatment might provide an indicator of the ultimate outcome and guide the physician whether to continue, stop, or adjust the dose of antibody. Most of the antibody was cleared within a few days of the final dose in the nonresponders. However, the half-life at the end of treatment in the responders was comparable with those previously reported for patients without bulky tumors.

Accumulation of alemtuzumab in the blood was significantly slower in the subcutaneous study and it took on average about 6 weeks longer to reach 1.0 μg/mL (an arbitrary threshold known to be potentially lympholytic). This could be a slight overestimate because samples were not always collected every week. Nevertheless, the eventual maximum trough concentrations were very similar in both studies. We cannot tell whether the slower accumulation of antibody in the blood was due to less favorable biodistribution, more effective binding to tumor cells, or the slower dose escalation used in the subcutaneous study compared with that used with intravenously treated patients. It should be possible to resolve this by studies of alemtuzumab given subcutaneously to patients not suffering from malignancies (ie, without excess $CD52^+$ cells).

Because clinical responses were much more frequent (19% CR and 68% PR),[8] we could not detect a significant correlation between blood concentrations and clinical responses in the subcutaneous study. With only 2 nonresponders in the group of patients we analyzed, the sample was too small to discern any relationship.

Clear-cut results were obtained from the measurements of antiglobulin responses (antihuman antibody [HAHA]). There were no detectable responses at all in the intravenous study (heavily pretreated patients) and only one patient in the subcutaneous study (previously untreated patients) made a strong response, reaching a peak of approximately 3.7×10^6 U/mL 3 weeks after the final dose. This was equivalent to 3.7 mg/mL of the monoclonal anti-idiotype standard and represented a substantial proportion of the total Ig in the patient. Similar titers were previously reported during treatment of rheumatoid arthritis patients with subcutaneous alemtuzumab[13] when all of the patients (10/10) made a positive response. After screening 11 other patients we found one with a significant antiglobulin titer of 4800 U/mL. Considering that this sample was taken 32 months after the last treatment with alemtuzumab, it seems likely that this patient also had made a very strong response.

Previous studies on antiglobulin responses to alemtuzumab have given widely different results. In 12 patients treated for kidney transplant rejection no antiglobulins were detected, in contrast to 15 of 17 patients treated with the parental rat antibody CAMPATH-1G.[11] In 167 patients treated for chemotherapy-resistant lymphoid malignancies there were only 3 weak or borderline responses. However, out of 115 patients treated for rheumatoid arthritis, there were 59 positive responses (ILEX Pharmaceuticals, unpublished data on file, July 2003), some of high titer similar to that reported here for patient no. 8036.[13] Many factors could affect the immunogenicity of alemtuzumab, including the dose and length of treatment, the underlying disease, prior exposure to chemotherapy, concomitant immunosuppressive drugs, and possibly the route of administration or the formulation of the drug. Subcutaneous administration might allow more effective antigen presentation, for example, by Langerhans cells and with less "excess" antibody circulating to provide tolerogenic signals, the balance might be tipped toward an immune response. From our present study we cannot say whether subcutaneous administration in CLL is more immunogenic than intravenous administration since there were other differences between the 2 groups, most notably the subcutaneous group had received no prior chemotherapy. Even if the 2 groups had been comparable, the difference in responses (0/30 vs 2/32) did not reach statistical significance. This issue needs to be investigated in larger numbers of patients. Both patients who made an antiglobulin response had been treated with alemtuzumab manufactured at the Therapeutic Antibody Centre. We are aware that the manufacture, formulation, or storage of biologics might affect their immunogenicity, but so far we have no evidence to suggest that there is a major difference between the academic or commercial source since antiglobulin responses have been reported in other patients treated with alemtuzumab from either source. The 2 patients (no. 8020 and no. 8036) who produced anti–alemtuzumab antibody on subcutaneous treatment, unlike the other patients, did not show significant reductions in lymphocyte counts but had marked local skin reactions which did not diminish with continued therapy. An antiglobulin response should be considered when there is no drop in the blood lymphocyte count and/or there are persistent injection site reactions.

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Alemtuzumab provides a useful therapeutic option for patients with CLL. It has excellent activity as first-line treatment and can give good responses in patients who are unresponsive to conventional chemotherapy. The optimal dose route and regimen are not yet known. However, the subcutaneous route can deliver concentrations of antibody comparable to the original intravenous route. The dominant factor influencing biodistribution and pharmacokinetics appears to be the tumor burden. With intravenous dosing, serum concentrations of alemtuzumab can provide an early prediction of the ultimate response and might be useful to guide dose adjustments to improve responses or reduce side effects. Strong anti-idiotype responses may occur in a small minority of patients. Although not associated with serious adverse effects, such responses neutralize the therapeutic benefit and therefore patients with a known antiglobulin response should not be further treated with this monoclonal antibody.

Acknowledgments

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T1897/14-3.3.04
(05 71 2607.0 / 1720571)
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Our Ref.: M2367 OPP(EP)

DRUG NAME: Alemtuzumab

SYNONYM(S): Campath, Ldp 103, Mabcambath, MabCampath-1H

COMMON TRADE NAME(S): MABCAMPATH®

CLASSIFICATION: monoclonal antibody

Special pediatric considerations are noted when applicable, otherwise adult provisions apply.

MECHANISM OF ACTION:

Alemtuzumab is a recombinant human IgG-derived monoclonal antibody that binds to antigen CD52 which is found on the surface of B and T lymphocytes, most monocytes, macrophages and NK cells, and certain granulocytes, but not hematopoietic stem cells.[1] Lysis of CD52-positive cells occurs via complement activation, antibody-dependent cytotoxicity, and apoptosis.[2]

PHARMACOKINETICS:

Table refers to intravenous (IV) dosing. Subcutaneous (SC) administration of alemtuzumab achieves concentrations similar to those with intravenous alemtuzumab, although with a slightly higher cumulative dose.[3,4]

Distribution	cross blood brain barrier?	no information found
	volume of distribution	mean 0.18 L/kg (range 0.1-0.4 L/kg)
	plasma protein binding	no information found
Metabolism	no information found	
	active metabolite(s)	no information found
	inactive metabolite(s)	no information found
Excretion	no information found	
	terminal half life	mean 11 h (range 2-32 h) after the first dose; 6 d (range 1-14 d) after 12 weeks
	clearance	wide variability, affected by tumour burden[5]; clearance decreases with repeated administration due to decreased receptor mediated clearance (loss of CD52 receptors in the periphery)
Elderly	comparable AUC in patients older than 65 years of age	

Adapted from standard reference[1] unless specified otherwise.

USES:

Primary uses:
*B-cell chronic lymphocytic leukemia[1,6]
T-cell prolymphocytic leukemia[6-8]

Other uses:
Cutaneous T-cell lymphoma[7]
Peripheral T-cell lymphoma[7]

*Health Canada approved indication

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SPECIAL PRECAUTIONS:

Contraindicated in patients who have a history of hypersensitivity reaction to alemtuzumab, active infections, underlying immunodeficiency (e.g., seropositive for HIV), or other active malignancies.[1]

Caution:

- in patients with previous hypersensitivity or anaphylactic reactions to other monoclonal antibodies[9]
- gradual dose escalation and appropriate pre-medication is required; for more information, see paragraph following the **Side Effects** table[1]
- single doses of alemtuzumab greater than 30 mg or cumulative doses greater than 90 mg per week should not be administered as they are associated with a higher incidence of pancytopenia[1]
- transfusion-related graft-versus-host-disease can occur in patients with severe lymphopenia and can be life threatening; patients receiving alemtuzumab should receive irradiated blood products, effectively eliminating this risk[1]
- *Pneumocystis* pneumonia (PCP) and herpes virus prophylaxis are recommended upon initiation of therapy[1,6,10,11]
- use alemtuzumab with caution in patients with recent exposure to the chicken pox virus[9]

Hepatitis B (HBV) reactivation: All lymphoma patients should be tested for both HBsAg and HBcAb. If either test is positive, such patients should be treated with lamivudine 100 mg/day orally, for the entire duration of chemotherapy and for six months afterwards.[6,10] Such patients should also be monitored with frequent liver function tests and HBV DNA at least every two months. If the HBV DNA level rises during this monitoring, management should be reviewed with a hepatologist and consideration given to halting chemotherapy.[6]

Cytomegalovirus reactivation: for more information, see paragraph following the **Side Effects** table

Carcinogenicity: no information found[1]

Mutagenicity: no information found[1]

Fertility: Alemtuzumab has been shown to bind to mature sperm.[1] No long term studies have been performed on men or women to determine the effect on infertility. Patients of reproductive potential should use effective contraceptive methods during treatment and for a minimum of 6 months following alemtuzumab therapy.[1]

Pregnancy: FDA Pregnancy Category C.[7] Either studies in animals have revealed adverse effects on the fetus (teratogenic or embryocidal or other), and there are no controlled studies in women, or studies in women and animals are not available. Alemtuzumab should be given only if the potential benefit justifies the potential risk to the fetus. Human IgG is known to cross the placental barrier, therefore alemtuzumab may also cross the placenta and cause fetal B and T lymphocyte depletion.[1]

Breastfeeding should be discontinued during treatment and for at least 3 months following the last dose of alemtuzumab as human IgG is excreted in breast milk.[1]

SIDE EFFECTS:

The table includes adverse events that presented during drug treatment but may not necessarily have a causal relationship with the drug. Because clinical trials are conducted under very specific conditions, the adverse event rates observed may not reflect the rates observed in clinical practice. Adverse events are generally included if they were reported in more than 1% of patients in the product monograph or pivotal trials, and/or determined to be clinically important.[12] When placebo-controlled trials are available, adverse events are included if the incidence is $\geq$ 5% higher in the treatment group.

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Table refers to IV data unless specified. SC hematological and infectious complications are similar to IV.

ORGAN SITE	SIDE EFFECT
Clinically important side effects are in ***bold, italics***	
allergy/immunology	anaphylactic reaction (<1%)[13] first dose reactions, infusion-related reactions with SC use, injection site reactions and fever
	angioedema (<1%)[13]
	antibody development (2%)
	autoimmune diseases; Graves' disease, Guillain-Barre syndrome, hypothyroidism, Goodpasture's syndrome, autoimmune thyroiditis[14]
	mouth edema (1%, severe 1%)
blood/bone marrow/ febrile neutropenia	anemia (77-80%, severe 38%)[13]; autoimmune hemolytic anemia (1%)[13]
	atypical lymphocytes (1%, severe 1%)
	immunosuppression,[1,15] ***lymphopenia (100%)***[15]***; onset in 2-4 weeks, may persist for over one year***[10]
	neutropenia (85%, severe 64%)[13]***; during the first weeks of therapy,***[15] ***nadir 4-6 weeks,***[15] ***median duration 21 days***[13]***; febrile neutropenia (5-10%, severe 1%)***[13]
	pancytopenia (5%, severe 3%),
	thrombocytopenia (72%, severe 50%) nadir 2-4 weeks,[15] ***median duration 21 d (range 2-165 d);*** autoimmune idiopathic thrombocytopenia (2%)
cardiovascular (arrhythmia)	arrhythmias (<1%)[13]
	tachycardia (5-11%, severe 1%)[13]
cardiovascular (general)	cardiac ischemia, infarction (<1%)[13,16,17]
	hypotension (30-32%, severe 3%)[13]; with SC use (0%)[3]
	hypertension (9-11%, severe 1%)[13]
coagulation	disseminated intravascular coagulation (<1%)[13,18]
	deep vein thrombosis, pulmonary embolism (<1%)[13]
	thrombocytopenic purpura (2%, severe 0%)
constitutional symptoms	asthenia (9%, severe 0%), malaise (5%, severe 0%), fatigue (27-34%, severe 4%)[13]; with SC use (5%, severe 2%)[3]
	fever (82-85%, severe 14%)[13]; usually occurs 5-6 hours after the start of the infusion[19] with SC use (70%, severe 2%)[3]
	insomnia (1-10%, severe 0%)[13]
	rigors (85%, severe 15%); with SC use (17%, severe 2%)[3]
	sweating (15%, severe 1%)
	temperature change sensation (5%, severe 0%)
	weight loss (5%, severe 0%)
dermatology/skin	*extravasation hazard: none*[20]
	bullous eruptions (1%, severe 1%)
	erythematous rash (4%, severe 1%)
	flushing (4%, severe 0%)

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ORGAN SITE	SIDE EFFECT
Clinically important side effects are in ***bold, italics***	
	injection site reaction (1%, severe 0%) with SC use (90%, severe 2%)[3]; onset approximately 24 h after the first dose, often persists until the fourth administration[21]
	pruritus/urticaria (21-30%, severe 1-5%)[13]; with SC use (0%)[3]
	rash (29-40%, severe 3%)[13]; with SC use (0%)[3]
endocrine	ADH secretion abnormality (<1%)[22]
	hyperthyroidism (<1%)[1]
gastrointestinal	*emetogenic potential: rare*[23]
	anorexia (13-20%, severe 1%)[13]
	ascites (<1%)[13]
	colitis, enteritis (<1%)
	constipation (2%, severe 0%)
	dehydration (2%, severe 2%)
	diarrhea (13-22%, severe 1%)[13]; with SC use (0%)[3]
	dyspepsia (4%, severe 0%); ulcer (<1%)[13]
	mucositis (2%, severe 0%)
	nausea (49-54%, severe 2%)[13]; late nausea is rare[15] with SC use (0%)[3]
	stomatitis (5%, severe 1%); ulcerative stomatitis (3%, severe 0%)
	taste loss (2%, severe 1%)
	vomiting (37-41%, severe 4%)[13]; with SC use (0%)[3]
hemorrhage	GI hemorrhage (<1%)[13]
	hemoptysis (1%, severe 0%)
	CNS hemorrhage (<1%)[13]
	purpura (8%)[13]
hepatobiliary/pancreas	liver dysfunction (1%, severe 0%)
	pancreatitis (<1%)[13]
infection	**bacterial** (34-40%)[10]; usually occurs within 8 weeks, mycobacterial and tuberculosis have occurred months later[10] bacteremia without sepsis (4%)[24] *Listeria monocytogenes* meningitis (1%, severe 1%) *Pneumocystis* pneumonia (PCP)
	fungal (18-21%)[10]; majority occur within 3 months of treatment, late infection have occured[10] *Aspergillosis* (2%, severe 1%) candidiasis (8-10%, severe 1%)[13] *Cryptococcal* pneumonia (1%, severe 1%) mucormycosis (2%, severe 2%) *Torulopsis* pneumonia (1%, severe 1%)

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ORGAN SITE	SIDE EFFECT
Clinically important side effects are in ***bold, italics***	
	not otherwise specified (7%, severe 1%)
	pneumonia (7-18%, severe 13%)[24]
	protozoan[7,24]
	sepsis (15-18%, severe 13%)[13]
	upper respiratory tract infection (6%, severe 0%)
	urinary tract infection (<3%, severe 0%)[24]
	viral (43-45%)[10] adenovirus[25] Cytomegalovirus (CMV) reactivation (4-66%)[3,10,14,15,24,26-28]; usually occurs between weeks 3-8 when T-cell numbers are low,[15] symptomatic CMV reactivations and infections (1-6%, severe 3%)[1,24,26,28] herpes simplex (10-13%, severe 1%)[13,24] herpes zoster (5%, severe 1%) Parvovirus B19 induced red cell aplasia (<1%)[29,30]
lymphatics	edema (1-13%, severe 0%)[13]
metabolic/laboratory	acidosis (<1%)[13]
	hypocalcemia (2%, severe 0%)
	hyponatremia (1%, severe 1%)
	increased alkaline phosphatase (<1%)[13]
	positive Coombs test without hemolysis (2%)
musculoskeletal	arthralgia (1%, severe 0%)
	myalgia (9-11%, severe 0%)[13]
neurology	anxiety (3%, severe 0%)
	confusion (2%, severe 1%)
	depression (2-7%, severe 0%)[13]
	dizziness (5-12%, severe 0%)[13]; vertigo (3%, severe 0%)
	leukoencephalopathy (<1%)[13]
	neurological symptoms associated with previous multiple sclerosis lesions; reversible, do not occur if pre-medicated with high dose steroids
	seizure (<1%)[13]
	sensory neuropathy; dysthesia (15%),[13] paresthesia (6%, severe 0%), hypoesthesia (3%, severe 0%)
	somnolence (3-5%, severe 0%)[13]
	tremor (6%, severe 0%)
ocular/visual	conjunctivitis (2%, severe 0%)
	endophthalmitis (1%, severe 1%)
	optic neuropathy (<1%)
pain	abdominal pain (6-11%, severe 2%)[13]

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ORGAN SITE	SIDE EFFECT
Clinically important side effects are in ***bold, italics***	
	back pain (5%, severe 2%)
	chest pain (6-10%, severe 1%)[13]
	headache (18-24%, severe 1%)[13]; with SC use (0%)[3]
	SC injection site pain (61%, severe 2%)[3]
	not otherwise specified (7%, severe 1%)
	skeletal pain (3-24%, severe 1%)[13]
pulmonary	bronchitis (9%, severe 1%)
	bronchospasm (6%, severe 2%)
	cough (4-25%, severe 1%)[13]
	dyspnea (18-26%, severe 6%)[13]; with SC use (0%)[3]
	hypoxia (2%, severe 1%)
	pharyngitis (4-12%, severe 0%)[13]
	pneumonitis (3%, severe 3%)
	pulmonary infiltration (1%, severe 1%)
	respiratory depression (<1%)
	rhinitis (1-7%, severe 0%)[13]
	sinusitis (6%, severe 1%)
renal/genitourinary	hematuria (1%, severe 1%)
	decreased renal function/renal failure (<1%)[13,18]
sexual/reproductive function	binds to mature sperm
syndromes	influenza-like symptoms (5%, severe 0%)
	tumour lysis syndrome (<1%)[13]
vascular	phlebitis (<1%)
	vasospasm (1%, severe 0%)

Adapted from standard reference[1] unless specified otherwise.

Infusion reactions: Intravenous administration of alemtuzumab is usually accompanied by transient infusion-related side effects that manifest as flu-like symptoms. These acute cytokine-mediated reactions are generally mild to moderate. The reactions includes nausea and vomiting, hypotension, dizziness, rigors, fever, diarrhea, sudden sweating, shortness of breath, chills, and/or rash.[1,15] Serious and potentially fatal complications including myocardial infarction, cardiomyopathy, cardiac arrest, arrhythmias, bronchospasm, and acute respiratory distress have also been reported.[1,13] Symptoms are most common during the first week of therapy and decrease with each subsequent administration.[2,2,9,31] To prevent infusion related reactions:

- give diphenhydramine 50 mg PO and acetaminophen 650 mg PO 30 minutes prior to alemtuzumab[1,6]
- pre-medicate with prednisone 10 mg PO 15-30 minutes prior to alemtuzumab for the first 1-2 weeks[6,15]; since corticosteroids cause immunosuppression, prophylaxis should be discontinued after 1-2 weeks[15]
- start with low doses of alemtuzumab (3 mg) and escalate as tolerated[1] (for more information, see **Dosage Guidelines**); it is not known if the infusion reactions are strictly dose-related[5]

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- meperidine 25 mg IV may lessen rigors,[6,9] patients experiencing meperidine-induced nausea should receive antiemetics[19]
- for patients experiencing severe rash, premedication with H_2 receptor antagonists (e.g., ranitidine) is recommended[19]
- fever commonly begins 5-6 hours after the infusion starts; a second dose of acetaminophen may be required[19]

During the infusion, the patient's pulse, respiratory rate and blood pressure should be monitored every 15 minutes during the first hour, then every 30 minutes.[6] Patients should be observed for 1 hour after the infusion.[6] To treat severe infusion-related symptoms, stop the alemtuzumab infusion and give hydrocortisone 200 mg IV.[1] Beta-2 agonists for wheezing and volume support for hypotension may be given.[32] If acute infusion reactions persist, the infusion time may be extended to up to 8 hours from the time of dose preparation.[31] Mild first-dose side effects tend to not preclude patients from completing therapy.[32]

Subcutaneous administration of alemtuzumab reduces infusion-related symptoms, while maintaining the same efficacy and improving convenience.[33] The incidence of nausea and vomiting, rigors, rash, hypotension, and dyspnea are significantly reduced with SC dosing, though fever may not be reduced appreciably (see Side Effects Table).[3] Almost all patients receiving SC alemtuzumab will develop a transient grade 1 or 2 local skin reaction.[3] IV corticosteroids do not affect the intensity or duration of these skin reactions.[3] Ice packs applied to the injection site 15 minutes prior to and 15 minutes post injection may decrease the severity of local skin reactions. Cold compresses applied to the injection site for 15-20 minutes, several times a day after treatment may also help alleviate symptoms. Most SC first-dose reactions disappear after 1-2 weeks.[3] Patients should be observed for 1 hour after the dose has been administered until at least three 30 mg doses have been given.[6] After that, monitoring may be discontinued.[6] Data from an ongoing study suggests that dose escalation may not be necessary with SC administration.[15]

Immunosuppression / opportunistic infections: Patients are at risk for opportunistic infections due to the profound lymphopenia induced by alemtuzumab.[1] Alemtuzumab-induced lymphopenia is non-dose dependant; single doses may reduce CD4+ and CD8+ T-cells for months.[15] Serious and sometimes fatal bacterial, fungal, viral and protozoal infections have occurred with infection rates ranging from 23-79% in pre-treated patients.[1,10] Although the majority of infections occur within 3 months of starting treatment, infections have been reported up to 6 months later.[10] If an infection occurs, alemtuzumab therapy should be interrupted but may be reinitiated following the resolution of the infection.[1]

Upon initiation of alemtuzumab patients should receive prophylaxis against PCP and herpes virus infections with:

- trimethoprim/sulfamethoxazole (TMP/SMX) DS PO twice daily three times weekly (TMP/SMX is the preferred prophylaxis; dapsone, pentamidine and atovaquone have also be used[10]
- acyclovir 400 mg PO BID (alternatively, valacyclovir 500 mg PO twice daily)[3]

Prophylaxis should be continued for a minimum of 2 months following the last dose of alemtuzumab or until CD4+ counts are ≥200 cells/μL, whichever occurs later.[1] Full recovery of CD4+ and CD8+ counts may take more than 12 months[1]; optimal length of post-treatment prophylaxis has yet to be determined.[10,15,19] Prophylaxis against PCP and herpes may decrease, but not eliminate, the occurrence of these infections.[1] Routine antifungal prophylaxis is not currently recommended but may be considered for high risk patients.[10,26]

Cytomegalovirus reactivation is the most common infection associated with alemtuzumab. T-cell depletion increases the risk of reactivation of CMV.[15,19] Reactivation usually occurs 3-8 weeks after initiation of therapy and coincides with T-cell nadir. Patients receiving alemtuzumab should have weekly testing for CMV replication by PCR or CMV antigenemia (pp65ag) assays.[6,11,19] Patients should also be monitored for symptoms of CMV infection.[6,19,34] If a positive screening test occurs, alemtuzumab treatment should be interrupted until testing becomes negative. Patients who are symptomatic should be treated immediately with ganciclovir.[10,14,15,19] Alemtuzumab has been restarted after clinical symptoms resolve and PCR testing is negative without further reactivation.[3,14,15,19]

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INTERACTIONS:

AGENT	EFFECT	MECHANISM	MANAGEMENT
diagnostic serum tests that utilize antibodies[1,13]	inaccurate test results[1] and potential for increased risk of allergic reactions[13]	monoclonal antibodies may result in the formation of human antichimeric antibodies (HACA); the presence of HACA may increase the risk of hypersensitivity reactions when other monoclonal antibodies are administered[13]	interpret with caution
monoclonal antibodies[1,35]	increased risk of allergic or hypersensitivity reactions to monoclonal antibodies; especially in patients that experienced previous hypersensitivity reactions to monoclonal antibodies[1]	monoclonal antibodies may result in the formation of human antichimeric antibodies (HACA); the presence of HACA may increase the risk of hypersensitivity reactions, thrombocytopenia, or diminished therapeutic response when other monoclonal antibodies are administered; monoclonal antibodies that also form HACA may be of particular concern	monitor for toxicity/response, patients who developed hypersensitivity to alemtuzumab may develop hypersensitivity reactions to other monoclonal antibodies
killed virus vaccines[1]	ability to respond to vaccines following therapy is unknown; duration of decrease response unknown; estimates vary from 3 months - 1 year[9]	alemtuzumab may decrease the ability to generate a humoral response to vaccines	immunize prior to therapy if possible, potential for decreased benefit of vaccine if administered during or within 1 year after therapy
live virus vaccines[1]	ability to respond to vaccines following therapy is unknown, risk of infection by the live vaccine virus; duration of risk unknown; estimates vary from 3 months - 1 year[9]	alemtuzumab may potentiate replication of the vaccine virus, decrease the ability to generate a humoral response to the vaccine and enhance the adverse effects of live vaccines[9]	avoid during and within 1 year after therapy

SUPPLY AND STORAGE:

For basic information on the current brand used at the BC Cancer Agency, see Chemotherapy Preparation and Stability Chart in Appendix.

SOLUTION PREPARATION AND COMPATIBILITY:

For basic information on the current brand used at the BC Cancer Agency, see Chemotherapy Preparation and Stability Chart in Appendix.

Additional information: *Diluted solution for infusion*: compatible with PVC bags and administration sets[1]

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Compatibility: consult detailed reference

PARENTERAL ADMINISTRATION:

BCCA administration guideline noted in ***bold, italics***

Subcutaneous	***rotate sites on thighs or abdomen****
Intramuscular	no information found
Direct intravenous	contraindicated
Intermittent infusion	***over 2 hours***
Continuous infusion	no information found
Intraperitoneal	no information found
Intrapleural	no information found
Intrathecal	no information found
Intra-arterial	no information found
Intravesical	no information found

*Subcutaneous administration of alemtuzumab significantly reduces flu-like symptoms making it the preferred route of administration.[12]

DOSAGE GUIDELINES:

Refer to protocol by which patient is being treated. Numerous dosing schedules exist and depend on disease, response and concomitant therapy. Guidelines for dosing also include consideration of absolute neutrophil count (ANC). Dosage may be reduced, delayed or discontinued in patients with bone marrow depression due to cytotoxic/radiation therapy or with other toxicities.

Adults:

BCCA usual dose noted in ***bold, italics***

Intravenous:

Cycle Length: ***titration 1 week***[1,6]***:***

3 mg IV for one dose on day 1, then 10 mg IV for one dose on day 3, and 30 mg IV for one dose on day 5

- **titration may be handled more slowly if required (e.g., infusion-related toxicities > NCI Grade 2)**
- gradual dose escalation is required at the initiation of therapy and after treatment interruptions of 7 days or more; dose escalation to the recommended maintenance dose of 30 mg is required and can be accomplished in 3-7 days in most patients
- doses greater than 30 mg per day or greater than 90 mg per week are not recommended

maintenance 1 week[1,6]***:***

30 mg (range 3-30 mg) IV for one dose on days 1, 3 and 5 (total dose per cycle) 90 mg [range 9-90 mg])

- consecutive daily dosing may also be used[1]
- gradual dose escalation is required after treatment interruptions of 7 days or more

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BCCA usual dose noted in ***bold, italics***

Subcutaneous:

Cycle Length:

titration 1 week[3,6]***:*** ***3 mg SC for one dose on day 1, then 10 mg SC for one dose on day 3, and 30 mg SC for one dose on day 5***

- **titration may be handled more slowly if required (e.g., infusion-related toxicities > NCI Grade 2)**
- gradual dose escalation is required at the initiation of therapy; dose escalation to the recommended maintenance dose of 30 mg is required and can be accomplished in 3-7 days in most patients
- doses greater than 30 mg per day or greater than 90 mg per week are not recommended

maintenance 1 week[3,6]***:*** ***30 mg (range 3-30 mg) SC for one dose on days 1, 3 and 5 (total dose per cycle 90 mg [range 9-90 mg])***

- consecutive daily dosing may also be used[1]

Concurrent radiation: additive bone marrow suppression may occur; dose reduction may be required when used concurrently or consecutively[9]

Dosage in myelosuppression:[1] modify according to protocol by which patient is being treated; if no guidelines available, refer to the table below

ANC ($x10^9$/L)		Platelets ($x10^9$/L)	Dose
< 0.25	or	≤ 25	**first occurrence:** hold treatment and resume at the same dose when ANC ≥ 0.5 and platelets ≥ 50 (restart at 3 mg if delay between doses ≥ 7 days and escalate to 30 mg as tolerated) **second occurrence:** hold treatment and resume at 10 mg when ANC ≥ 0.5 and platelets ≥ 50 (restart at 3 mg if delay between doses ≥ 7 days and escalate to a maximum dose of 10 mg) **third occurrence:** discontinue treatment permanently
For baseline ANC ≤ 0.5 and/or platelets ≤ 25: if ANC or platelets decrease to ≤ 50% of baseline value, hold therapy and resume when ANC and platelet count return to baseline values (restart at 3 mg if delay between doses ≥ 7 days and escalate to 30 mg as tolerated)			
There is no information on the safety of resumption of alemtuzumab in patients with autoimmune cytopenias or marrow aplasia.[1]			

Dosage in renal failure: no information found

Dosage in hepatic failure: no information found

Dosage in dialysis: no information found

Children: has been used[36]

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Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Our Ref.: M2367 OPP(EP)

EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

15 July 2010
EMA/HMPC/16633/2009
Committee on Herbal Medicinal Products (HMPC)

Assessment report on *Vitis vinifera* L., folium

Based on Article 10a of Directive 2001/83/EC as amended (well-established use)

Based on Article 16d(1), Article 16f and Article 16h of Directive 2001/83/EC as amended (traditional use)

Final

Herbal substance(s) (binomial scientific name of the plant, including plant part)	*Vitis vinifera* L., folium
Herbal preparation(s)	Herbal substance - Not applicable Herbal preparations: - Comminuted dried leaves as herbal tea (TU) - Powdered herbal substance (TU) - Soft extract (2.5-4:1; water (TU) - Dry extract (4-6:1; water) (WEU)
Pharmaceutical forms	Comminuted herbal substance as herbal tea for oral use (TU). Herbal preparation in solid dosage forms for oral use (WEU and TU). Herbal preparation in semi-solid dosage forms for cutaneous use (TU).
Rapporteur	Dr Ioanna Chinou, Gloria Garcia-Llorente

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1. Introduction

The aim of this report is to assess the preclinical and clinical available data on Vitis viniferae folium for preparing a Community herbal monograph and Community List entry. This report is based on the documentation provided by the European Medicines Agency (EMA) completed by additional searches and information taken from recent international literature on Vitis viniferae folium.

Bibliographic searches have been performed in MEDLINE (1966-2008); EMBASE (1974-2008). The search terms were: Plant extracts; Plant leaves; Red vine leaf extract; Roter Weinlaubextrakt; Extract Red vine / wine Leaf / Leaves *Vitis vinifera*; Folia *Vitis vinifera.*
Only preparations of *Vitis vinifera* as a single ingredient are considered for the monograph, while the studies performed with combinations are not discussed in this report.

1.1. Description of the herbal substance(s), herbal preparation(s) or combinations thereof

The plant originates from North Africa, South Africa or South West Europe. *Vitis vinifera* L., Grape vine, is a perennial, defoliating limber with a wooden often twisted stem which can reach a length of 30 meters, but it is usually cut back to 1-3 meters. The shrub develops climbing branches forking to twigs from where the long-stemmed, alternating arranged leaves protrude. The vine leaf is heart-shaped, thin, long-stemmed, cordate with palmate venation and with 5-7 dentate lobes, divided by more or less deep and open sinuses. It can be up to 15 cm long and 12 cm across at its widest point, its colour is a uniform dark red. The upper surface is glabrous, but the lower surface may be pubescent. The venation is prominent on the lower surface. At the lower tendrils the flower panicles with numerous yellow-greenish flowers are formed. The fruits, arranged in large and long clusters are soft and pulpy berries with yellow-green, reddish or purplish dark-blue skin [Pharm. Franc. X., 1996].
Vitis vinifera belongs to the *Vitaceae* family. Several subspecies and varieties are distinguished among which is the subspecies *sylvestris* (Gmelin) Berger, recognised as the spontaneous form of *V. vinifera* L., the subspecies *caucasica* Vavilov, occurring in both wild and cultivated form. It is supposed that from these two, the cultivated form *Vitis vinifera* ssp. *sativa* DC has been grown [Bombardelli & Morazzoni 1995, Bombardelli et al. 1997].

- Herbal substance

The herbal substance consists of the dried leaves of the black to pulp-red grape and has a faintly perceptible odour. The herbal drug is harvested by hand in the autumn following the grape harvest. Drying takes place under natural conditions in accordance with local climatic conditions.

Latin Name: Vitis viniferae folium (*Vitis vinifera* L., var. *tinctoria*, *Vitaceae*) "Vitis vinifera" or "Vitis folium";
Common Names: vine leaves or vineleaves or vine leaf or vineleaf or grape leaf (English);
Feuilles de vigne (French), Rebenblätter, Weinlaub (German), Fogli della vite (Italian), Hogas de la vid (Spanish), Folhas da videira (Portuguese), Wijnstok bladeren (Dutch), liść winorośli właściwej (Polish), Φύλλο Αμπέλου (Greek).

The crude herbal substance complies with the monograph "Vigne Rouge" French Pharmacopoeia [Pharm. Franc. X., 1996]. The powder is reddish-brown. Examined under the microscope, the powdered red vine leaf shows the following characteristics: more or less dense, unicellular, long, tapering, covering trichomes, thick-walled, with bulbous or truncated base and a lumen divided into loculi; numerous raphides of calcium oxalate are contained in these cells or scattered about; fragments

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of parenchyma containing twinned crystals of calcium oxalate; a few fragments of epidermis with polygonal cells and some reticulate venation.
Since the beginning of the last century in many studies the chemical constituents of the different parts of grape vine have been investigated. Fruit acids, tannins and pigments are the substances mainly responsible for taste, odour and colour [PDR for Herbal Medicines, 2004].

- Herbal preparation(s)

Comminuted herbal substance

Powdered herbal substance

Soft extract (DER 2.5-4:1); extraction solvent water

Dry extract (4-6:1; water)

See also market overview in 1.2

- Combinations of herbal substance(s) and/or herbal preparation(s) including a description of vitamin(s) and/or mineral(s) as ingredients of traditional combination herbal medicinal products assessed, where applicable.

Not applicable.

1.2. Information about products on the market in the Member States

Member State	**Regulatory Status**	**Comments**[1]
Member state	products, indications	Legal status
Austria	1) **360 mg-filmcoated tablet** 1 tablet contains: 360 mg dry extract (DER 4-6:1), extraction solvent water 2) **capsules** 1 capsule contains: 180 mg dry extract (DER 4-6:1), extraction solvent water 3) **oral drops** 1 ml contains 73.5 mg extract (corresponding 1.2 mg Aesculin) in ethanolic solution (the DER and the extraction solvent are not declared in the SPC at the moment) Since 1) December 2005 2) January 2000 3) March 1993 1) 1 tablet daily (up to 2 tablets). Duration of use: max. 3 months 2) 2 capsules in the morning (up to 4 capsules). Duration of use: max. 3 months 3) 30 drops in the morning in fasting state **Indications** 1,2) chronic venous insufficiency grade I and II; swelling of feet and lower leg	WEU

[1] Not mandatory field

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Member State	Regulatory Status	Comments[1]
	3) phlebitis, edemas in the legs (also during pregnancy), varicose veins	
Belgium	**Hard-capsules** 350 mg powder per capsule powder, corresponding to 8% total polyphenols and minimum 0.20% of anthocyanosides Since 2005 oral administration Adults: 1 to 2 capsules three times daily, during the meals with a large glass of water. Maximal dose is 6 capsules per day. Duration of use should be limited to 3 months. If symptoms do not improve during use, consult a doctor **Indications** used in case of subjective symptoms of venous insufficiency in legs, such as heavy legs, after exclusion of all serious pathologies such as phlebitis, thrombophlebitis and thrombosis	WEU
Bulgaria	Not known	
Cyprus	Not known	
Czech Republic	**(WEU)** Vitis viniferae folii extractum aquosum siccum (4–6:1), 180 mg – standardized to flavonoids content 3-7% **Pharmaceutical form** Capsule **Posology** 2 capsules corresponding to 360 mg of the extract in the morning, during first 3 weeks increase of the dose to 4 capsules is possible to reach faster relief since 2001?? **Indications** Prevention and therapy of symptoms of light or starting moderate forms of chronic venous insufficiency related to varicose veins such as swelling of the calves, heavy legs, tingling	
Denmark	No authorized herbal medicinal products containing *Vitis vinifera* as a single drug preparation are on the market.	
Estonia	Not known	
Finland	Not known	
France	**(WEU)** Soft extract since 1970 **Pharmaceutical form** eye drops **Posology** 1 drop 2 to 8 times daily. 1 g of extract/100 ml **Indications** uninfectious conjonctival irritations **TU** **Preparations** Powder since 1982 dry aqueous extract since 1987, 1990 All for oral use, in adults **Posology**	

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Member State	Regulatory Status	Comments[1]
	1) 1 hard capsule 3 times daily (5 if necessary) 350 mg of powdered drug/capsule 2) 1hard capsule 2 times daily 200 mg of extract/capsule 3) 1 hard capsule 1 to 3 times daily containing 169 mg of extract/capsule **Indications** 1); 2); 3) - Traditionally used in the symptomatic treatment of functional disorders of cutaneous capillary fragility, such as ecchymosis, petechias, etc. - Traditionally used:, in subjective signs of venous insufficiency, such as heavy legs - in haemorrhoidal symptoms combination herbal preparations with Mélilot (*Melilotus*) Marron d'inde (*Aesculus hippocastanum*) Hamamélis (Hamamelis)	
Germany	**TU** **Preparations**: soft extract (2.5-4:1), extraction solvent: water at least since 1976 **Pharmaceutical form** cream **Posology** for cutaneous use in adults 10 g cream contain 282 mg soft extract **Indications** Traditionally used to relieve symptoms of tired legs **Risks** (adverse drug effects, literature) Allergic reactions, itching and erythema, worsening of skin irritation. Contact allergy and/or hypersensitivity reactions **WEU** 1) soft extract (4-6:1), extraction solvent: water 2), 3) dry extract (4-6:1), extraction solvent: water In the market 1), 3) at least since 1976 2) 2005 1) oral liquid 2) film-coated tablet 3) capsule, hard all for oral use in adults **Posology** 1) 1 x daily 6 ml liquid if necessary 1 x daily 12 ml liquid 10 ml (= 11.013 g) liquid contain 0.6 g soft extract 2) 1 x daily 1-2 containing 360 mg dry extract each 3) 1 x daily 2 containing 180 mg dry extract each if necessary 1 x daily 4 **Indications** For symptomatic treatment of chronic venous insufficiency, which is characterised by swollen legs, a feeling	

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Member State	Regulatory Status	Comments[1]
	of heaviness, pain, itching, cramps in the calves at night **Risks** Common gastrointestinal complaints, nausea. Hypersensitivity reactions of the skin (itching and erythema, urticaria) have been reported	
Greece	No authorized herbal medicinal products containing *Vitis vinifera* as a single drug preparation are on the market	
Hungary	1)180 mg capsules 1 capsule contains: 180 mg dry extract (DER 4-6:1), extraction solvent water (2008) 2) oral drops 81.4 mg Vitis viniferae rubrae folii extr. spissum(4-6:1), corresponding to 1.2 mg aescin/ml (1997) 3) Varixinal capsules 180 mg dry native extract of *Vitis vinifera* L., folium (DER: 4-6:1) extraction solvent: water (2009) 1) 2 capsules once a day 2) 30 drops in the morning in fasting state 3) 2 capsules once a day **Indications** 1) chronic venous insufficiency; at stage of C_2, C_3 and C_{4A} according to CEAP 2); 3) For alleviating the discomfort feeling (fatigability, tension of the legs) of the lower extremities occurring in the course of the mild venous circulation disturbance 1); 3) It is not recommended during pregnancy and lactation. Adverse effects: Gastrointestinal disorders (Common) stomach and intestinal discomfort, nausea Skin and subcutaneous tissue disorders: (Uncommon) rash, itching, hives	
Iceland	Not known	
Ireland	Not known	
Italy		Food supplements only.
Latvia	**Preparations** *Vitis viniferae* folii extractum aquosum siccum (4-6:1) 180 mg - standardized to flavonoids content 3-7% since 2004 **Pharmaceutical form** Capsule **Indications** Prevention and treatment of signs related to chronic venous insufficiency	
Lithuania	Not known	
Luxembourg	Not known	
Malta	Not known	
Netherlands	Not known	

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Member State	Regulatory Status	Comments[1]
Norway	Not known	
Poland	Vitis viniferae extractum siccum (5-7:1), extraction solvent: water, capsule hard Product allowed onto the market according to previous polish pharmaceutical low (less than 10 years). **Posology** oral use: adults, 2 capsules once a day (in the morning) **Indications** support of mild symptoms of chronic venous insufficiency	
Portugal	No authorized herbal medicinal products containing *Vitis vinifera* as a single drug preparation are on the market	One authorised combination product.
Romania	Not known	
Slovakia	**Preparations** Vitis viniferae folii extractum aquosum siccum (4–6:1) 180 mg – standardized to flavonoids content 3-7% since 2005 **Pharmaceutical form** Capsule **Posology** 2 capsules (360 mg) once a day, in the morning time. Total daily dose can be increased up to 4 capsules once a day, in the morning time **Indications** 1) Prevention and treatment of signs related to chronic venous insufficiency, which is usually expressed in connection with varicoses and oedema in the lower parts of legs, fatigue, pins and needles and pain in legs **Risks (adverse drug effects)** Allergic reactions including generalized rash and urticaria. Nausea, gastric complaints	
Slovenia	Not known	
Spain	**Preparations** **(WEU)** Vitis viniferae folii extractum aquosum siccum (4–6:1), 180 mg – standardized to flavonoids content 3-7% **Posology** 2 capsules (360 mg) once a day, in the morning time. Since 2001 **Indications** Prevention and treatment of signs related to chronic venous insufficiency, which is usually expressed in connection with varicoses and oedema in the lower parts of legs, fatigue, pins and needles and pain in legs **(TU)** Powdered herb. substance 270 mg/caps	
Sweden	No authorized herbal medicinal products containing *Vitis vinifera* as a single drug preparation are on the market	
United Kingdom	Not known	

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Regulatory status overview

Member State	Regulatory Status				Comments (not mandatory field)
Austria	☒ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	(4-6.1, water extr tab, 2005, 180 mg caps, 2003, drops 73.5 mg/ ml, corresp. 1.2 (aesculin), 1993
Belgium	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	WEU Caps 350 mg 1- 6/d
Bulgaria	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known
Cyprus	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known
Czech Republic	☒ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Ext aq (4-6:1) standardized 3-7% flavonoids. Caps 180 mg, 2001
Denmark	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	No product registered
Estonia	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known
Finland	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known
France	☒ MA	☒ TRAD	☐ Other TRAD	☐ Other Specify:	WEU, eye drops Aqueous soft extract (6:1) since 1970 TU, powder(1989) or water extr. for oral use (1987)
Germany	☒ MA	☐ TRAD	☒ Other TRAD	☐ Other Specify:	dry water extr. (4-6:1), 180mg 1976, (360 mg) 2005 oral liquid, 10 ml contain 0.6 g soft extr. **TU** soft water extr. (2.5-4:1), 1976, cream, 10 g contain 282 mg soft extr.
Greece	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	No product registered
Hungary	☒ MA	☐ TRAD	☒ Other TRAD	☐ Other Specify:	(4-6.1, water extr tab, 2005, 180 mg caps, 2008 & 2009, drops 81.4 mg/ ml, corresponding. 1.2 (aesculin), 1997
Iceland	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known
Ireland	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known

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Member State	Regulatory Status				Comments (not mandatory field)
Italy	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Food Suppl. No product registered
Latvia	☒ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	WEU Ext aq (4-6:1) standardized 3-7% flavonoids caps 180 mg, 2004
Liechtenstein	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known
Lithuania	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known
Luxemburg	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known
Malta	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known
The Netherlands	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known
Norway	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known
Poland	☒ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	
Portugal	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	No product registered
Romania	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known
Slovak Republic	☒ MA	☐ TRAD	☒ Other TRAD	☐ Other Specify:	WEU Ext aq (4-6:1) standardized 3-7% flavonoids. Caps 180 mg, 2005
Slovenia	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known
Spain	☒ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	WEU Ext aq (4-6:1) standard. 3-7% flavon. caps 180 mg Powdered herb. substance 270 mg/caps
Sweden	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	No product registered
United Kingdom	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known

MA: Marketing Authorisation
TRAD: Traditional Use Registration
Other TRAD: Other national Traditional systems of registration
Other: If known, it should be specified or otherwise add 'Not Known'
This regulatory overview is not legally binding and does not necessarily reflect the legal status of the products in the MSs concerned.

2. Historical data on medicinal use

2.1. Information on period of medicinal use in the Community

Red grapevine leaves, from *Vitis vinifera* L. [Fam. *Vitaceae*], are rich in flavonoids including anthocyanins, oligomeric proanthocyanidins (OPCs) etc.
The knowledge of the medicinal potential of grape vine *(Vitis vinifera)* can be traced back far in history:

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- In Europe, the leaves of Vitis vinifera are documented in the literature of traditional medicine for their astringent and homeostatic properties where they are utilized in the treatment of diarrhoea, bleeding, haemorrhoids, varicose veins and other circulatory and venous diseases [Anonymous 2004; 2005; 2006; Bombardelli & Morazzoni 1995].
- In Turkish folk medicine, vine leaves are known to have a diuretic effect, while the juice of leaves have been used as an eye bath [Kosar et al. 2007].
- Native North American indigenous peoples used the leaf tea of related fox grape (*Vitis labrusca* L.) for treating diarrhoea, as well as for hepatitis, stomach aches and thrush and externally poulticed the wilted leaves for sore breasts, rheumatism, headaches and fevers. Other closely related Vitis species have been used similarly.
- In Indian Medicine: Grape is used for headache, dysuria, scabies, skin disease, gonorrhoea, haemorrhoids and vomiting [PDR for herbal Medicines 2004].

Since ancient times, beneficial effects on health have been ascribed to wine and vine leaves, as confirmed by numerous "recipes" reported in Egyptian papyri, the Sumerian tablets, the writings of Hippocrates of Cos (5th-4th century BC), Celsus (1st century AD), Galen (130-201 AD) and Paracelsus (1493-1541). The use of vine leaves has been documented. Retracing therapeutic literature from France showed the origin of using red leaves shortly after first incidence of the respective grape vine varieties called "teinturiers" [Schneider, 2007i]. The herbal substance and herbal preparations have a long history of use in folk medicine where French winegrowers collected the red vine leaves at the time of the grape harvest to make infusions and paste-like poultices from them. The infusions were filled into bottles and regularly ingested in small quantities. The paste from vine leaves was used for the treatment of swollen, painful legs. Nowadays, the extracts of grapevine leaves were developed into modern herbal medicaments mainly used against venous diseases and for its effects on microcirculation [Rabe et al. 2005; Volonté M, Petrini, 2004; Volonté et al. 2003]. The vine, fresh and brined or fermented are used as food (mixtures of rice and spices with or without meat are wrapped with vine leaves) and have been widely consumed as traditional foods (Dolmas) around the Mediterranean countries [Kosar et al. 2007]. They are also used for diarrhoea, vomiting and varicose treatment [Gharib Naseri & Heirari 2006].

There is evidence of the use of grapevine leaf outside France, in Italy in 1957, where the Biosedra preparation was also tested in vascular disorders in gynaecology, producing a positive effect on capillary fragility.

As early as 1960, a clinical study was published in Germany on the venous efficacy of a product, which contained a fluid extract of grapevine leaf, with a drug/extract ratio of 1:1. The content of anthocyanin was set at 600 µg/ml,which was subsequently changed to 1.2 mg bioflavonoids per ml (German *Rote Liste* 1974). In 1969, a company registered the preparation as a medicinal product, with the indications of varices, phlebitis, thrombophlebitis, calf cramps and leg oedema. The successor product, has been marketed since 1971. The capsules and tablets contain 180 mg and 360 mg of dried extract of grapevine leaves.

An overview of grapevine leaf components and its supposed pharmacological action as well as the polyphenol composition of *Vitis vinifera* can be found in the literature [Anonymous 2003; 2004; Petrini et al. 2003; Schneider, 2007i; 2007ii; Schneider et al. 2008; Schaefer & Red 2003].
Nonclinical *in vitro* and *in vivo* studies suggested protective effects of components from extracts of grapevine leaves on the venous system; e.g. procyanidines [Maffei et al. 1994; Constantini et al. 1999], and flavonoids [Nees et al. 2003]. For the extract preparation *(Extractum Vitis viniferae foliae aquosum siccum, 4-6:1)*, dried leaves of red varieties of *Vitis vinifera* L. (Vitis viniferae folium) which comply with the monograph described in Pharmacopée Français (10ème edition) for "vigne rouge" are

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used. Thus, the herbal substance consists of the dried leaves of the black to pulp-red grape which finally undergo a specific production process resulting in a defined flavonol content in the dry extract preparation. While the whole dry extract preparation as such is considered as active agent, it is particularly characterized by its content of flavonol glycosides and glucuronides, i.e. quercetin-3-O-β-D-glucuronide, quercetin-3-O-β-glucoside, and kaempferol-3-glucoside. These flavonoids are considered to contribute predominantly to pharmacological effects. The extract AS 195 contains a total of 4-7% of flavonol glycosides, quantified as quercetin-3-O-β-D-glucuronide.

Grapevine leaves and extracts thereof have been traditionally used for the treatment of symptoms associated with venous insufficiency for more than 70 years, in France. Extracts have been introduced in a number of European countries, e.g. Austria, Belgium, Czech Republic, Spain, Switzerland, Italy, and the United Kingdom. It is indicated for the prevention and treatment of Chronic venous insufficiency (CVI), associated with varicose veins including oedema of the lower leg, heavy or tired legs, sensation of tension, tingling and pain.

Based on the survey of products available in the Member states, the traditional use of red leaves preparations has been demonstrated especially in France, Germany, Spain and Austria, for at least 30 years.

Red vine leaf has been included in the French official list entitled "Avis aux fabricants concernant les demandes d'Autorisation de mise sur le marché" by virtue of which, the products containing it were subjected to a simplified registration procedure before the implementation of the Directive 2004/24/EC.
In France: "Vigne Rouge" (= red vine leaf) is regarded as one of the herbal drugs whose efficacy and safety has been proven by thorough literature studies and long-term traditional use. The Pharmacopèe Française X ed. includes the monographs of "Vigne rouge" and "Extrait de vigne rouge (sec)" and the traditional use of red vine leaves is discussed in "Rotes Weinlaub" [Schneider, 2007i; 2007ii].

Herbal medicinal products with red vine leaf have a long tradition in France. The official compendium "Médicaments à base de plantes", issued by the French department "Ministère des Affaires sociales et de la solidarité", released 1990, and comprised the long tradition of diverse herbals in France. There are listed many herbals that have been used in France for a long time, at least for about 15–20 years. [Les Cahiers de l'Agence 1998] state the use of red vine leaf for symptomatic relief of chronic venous disease ("No 015+016: " Traditionellement utilise dans le traitement symtomatique des troubles fonctionnels de la fragilité capillaire cutanée, tels que ecchymoses, pétéchies, etc."; No. 017+018: "Traditionnellement utilisée: - dans les manifestations subjectives de l'insuffisance veineuse telles que jambes lourdes; - dans la symptomatologie hémorroïdaire").
Different products prepared of water extracts of "Vigne Rouge" (5:1) have been available in oral forms, in France to treat blood vessels fragility in order to reduce the feeling of heavy legs and haemorrhoids related disorders. The posology range from 169 mg to 200 mg, two to three times a day, for four weeks:

Vigne rouge, capsules: 1 capsule contains 200 mg of grapevine leaves dry aqueous extract is used, a DER of about (4-6:1). The product is registered since 1987. The manufacturer recommends the use of 2 capsules per day (1 capsule mornings and one evening, with a glass of water). The product is traditionally used to reduce fine blood vessels fragility, in order to reduce the feeling of heavy legs and haemorrhoids related disorders.
OR
1 capsule contains 169 mg of grapevine leaves dry extract (3:1). The manufacturer recommends the use of up to 3 capsules per day. The product is traditionally used to reduce the feeling of heavy legs, haemorrhoids related disorder and fine blood vessels fragility.

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An article from 'Deutsches Medizinisches Journal' published in 1960 by K. Güthenke, "Wesen und Behandlung der Bindegewebsschwäche, insbesondere der Veneninsuffizienz mit Weinblattextrakt" (Nature and Treatment of weak connective tissues, in particular venous insufficiency, ref.) describes the long traditional use of grapevine leaves for treatment of diseases of the leg veins, and demonstrates that vine leaf extracts have been already traditionally used before 1960, in Germany. Registrations for the active ingredient *Extractum vitis viniferae foliae aquosum siccum* exist since 1969. Since 1974 a solution for oral use has been available. 20 ml contained 1-2 g of extract from leaves of *Vitis vinifera* (1:4-5) and 0.02 g of aesculin. In 1999 aesculin was removed from the formulation. The recommended dosage is 2-3 x 30 drops per day. The product is traditionally used to treat diseases of the leg veins (f.e. varicose veins, chronic venous insufficiency), to reduce pains and feeling of heavy legs, night time cramps in the calf, itching and swollen legs. The use of this product can be proven for over 30 years.

In 1991 the galenical form of hard gelatine capsules was registered and marketed for the first time. One capsule contained a combination of 180 mg of *Extractum vitis viniferae foliae aquosum siccum* and 3 mg aesculin (a coumarine glucoside that naturally occurs in the horse chestnut *Aesculus hippocastanum*). This herbal medicinal product was indicated for the treatment of venous insufficiency, varices, haemorrhoids, heavy tired legs and feet, essential oedema and calf cramps. The daily posology was 2-3 capsules a day. In 1999, aesculin was removed from the product and the only active substance declared was the dry extract of grapevine leaves. The capsules contain 180 mg of *Extractum vitis viniferae* (extraction solvent: water, DER: 4–6:1), with a recommended use of 2-4 capsules per day.
A cream (ointment) -containing soft extract (4-6:1; water) in a cream base
(10 g cream contain 282 mg soft extract)- has been traditionally used since 1976 to relieve symptoms of tired legs applying it several times daily.

Conclusions on traditional use
Based on information obtained from Member states and data retrieved from handbooks it can be concluded that the following extracts and uses of grapevine leaf extracts fulfil the criteria for traditional use:

Herbal substance

Not applicable.

Herbal preparations:

- Comminuted dried leaves
- Powdered dried leaf
- Soft extract (2.5-4:1; water) in a cream base

Concerning the preparation: dry extract (3:1; water), the product has been on the market since 1990 (France) and does not comply with the requirements of Directive 2004/24/EC for THMP. There is no complete information on the manufacturing process and DER of this preparation, and it is not possible to evaluate the similarity with other dry extracts under WEU.

2.2. Information on traditional/current indications and specified substances/preparations

Indication:

Traditional herbal medicinal product:

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a) To relieve symptoms of discomfort and heaviness of legs related to minor venous circulatory disturbances.

b) For symptomatic relief of itching and burning associated with haemorrhoids.

c) For symptomatic treatment of cutaneous capillary fragility.

The product is a traditional herbal medicinal product for use in the specified indication exclusively based upon long-standing use.

2.3. Specified strength/posology/route of administration/duration of use for relevant preparations and indications

Posology:

-Dried leaves in herbal teas, 40 g/l. One cup (10g/250 ml) 2-4 times/day [ESCOP 2009]
-Comminuted total powder: 270-350 mg, 3 times/day (before the meals), up to 2100 mg/day
-Soft extract (2.5-4:1; water), in a cream base, (10g cream contain 282 mg soft extract), a thin layer should be spread on the affected area 1-3 times/day

-Dry extract (DER 4-6:1; water), single dose: 360-720 mg, daily dose: 360-720 mg

3. Non-Clinical Data

Non-clinical *in vitro* and *in vivo* studies suggested protective effects of components from extracts of grapevine leaves on the venous system; e.g. procyanidines [Maffei et al. 1994; Constantini et al. 1999], and flavonoids [Nees et al. 2003].

3.1. Overview of available pharmacological data regarding the herbal substance(s), herbal preparation(s) and relevant constituents thereof

Constituents

Vine leaves contain a wide range of polyphenol flavonoids including flavon(ol)-glycosides and glucuronides, quercetin-3-O-beta-D-glucuronide (most abundant of flavonoids), isoquercitrin, anthocyanins, oligomeric proanthocyanidins [Hmamouchi et al. 1997], catechin, epicatechin monomers and dimmers and gallic acid. The phytoalexin trans-resveratrol, another polyphenolic substance belonging to the stilbene group, can also be found in grape vine [Langcake et al. 1979]. It has been referred in the international literature that $AlCl_3$ is a potent elicitor of resveratrol production in vine leaves [Andrian et al. 1996]. In vine leaves, also organic acids appear, mainly malic and oxalic acid but also tartaric acid. Citric, fumaric and succinic acid can be detected in the leaves only in traces. Compared to the grape berries, grape leaves are richer in the content of carotenoids and vitamin C. According to the French Pharmacopoeia, the dried leaves of grapevine should contain at least 4% of total polyphenols and 0.2% of anthocyanins [Pharm. Franc. X., 1996; Jonadet 1983; Lardos & Kreuter 2000; Laparra et al. 1989].

The leaves of the red varieties are very rich in tannins from the catechin group. The composition in tannins of leaves depends on the phase of development and on their position on the plant. In autumn, catechin, gallocatechin and epicatechingallate can be found in the leaves. From catechins and/or leukoanthocyanidines so called oligomeric proanthocyanidins, colourless substances, are formed. The greatest part of anthocyans consists of malvidin glucosides but also delphinidin, cyanidin and petunidin glucosides occur [Laparra et al. 1989]. The highest content of anthocyans can be detected in the red leaf especially in autumn, in the time between the vintage and the shedding of leaves [Raynaud, 2006,

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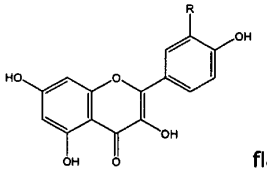
Darné & Glories 1988]. From a pharmacological point of view, the polyphenols, for example flavonoids, are the most important substance group [Bruneton 1999; 2002; Raynaud, 2005; Diaz Lanza et al. 1995].

Analytically the following chemical compounds have been determined in the leaves of *Vitis vinifera*:
- *Flavonoids* (up to 3.5% for red vine leaf, the content is higher in green leaves 4-5%): including kampferol-3-0-glucosides, quercetin-3-O-glucosides
- *Tannins*: procyanidolic oligomers (proanthocyanidins, about 4%) including constituents monomers of cathechin epicatechin
- *Stilbenes*: resveratrol and viniferins [Chung et al. 2003]
- *Fruit acids*: including tartaric acid, malic acid, succinic acid, citric acid, oxalic acid
- *Phenylacrylic acid derivatives*: p-coumaroyl acid, caffeoyl acid, feruloylsuccinic acid

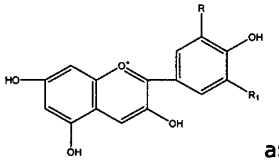
In a recent comparative study of 135 samples of grapevine leaves of different origin, the flavonol, anthocyanin and polyphenol contents have been determined. Total flavonol content was found to be between 0.6% and 3.5%, anthocyanin content between 0.2% and 1.45% and polyphenol content between 4.6% and 18.9%. HPLC methods used for determining anthocyanins and flavonols in grapevine leaves were validated and findings were compared to results produced by assays described in the French Pharmacopoeia. Whereas the correlation between conventional photometric and HPLC methods was satisfactory for anthocyanins, the correlation between the pharmacopoeia assay for total polyphenols and the HPLC analysis for flavonols was poor. As flavonol compounds are considered relevant for the vasoprotective effect of grapevine leaves, their content in starting material used in the production of herbal medicines needs to be quantified [Schneider et al. 2008].

Overview on main polyphenolic compounds in Grape Vine

Flavonoids	Flavones (Quercetin Kaempferol), Flavanes
Anthocyans	Leucoanthocyanidins, Anthocyanidins -responsible for the blue and red coloring of leaves, flowers and fruits -The concentration of anthocyans in the red colored leaf is high.
Catechins	• Grapevine leaf is rich in cathechins • Concentration of cathechins is dependent on: ->the leaf's position on the plant the phase of development of the leaf • In autumn cathechin, gallocathechin, epicathechingallat are present in the leaf.
Stilbenes	Resveratrol, *trans*-Resveratrol belong to the stilbenes (phenolic sub group) is a phytoalexin= stress-induced plant metabolite resveratrol can be found only in stressed leaves stress factors : i.e. fungal infection, UV-irritation, injury ->resveratrol is present in different forms depending on the stage of the plant's stress answer

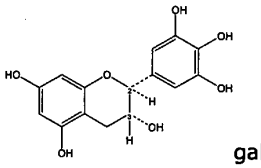


flavonol

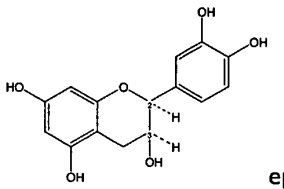


anthocyan

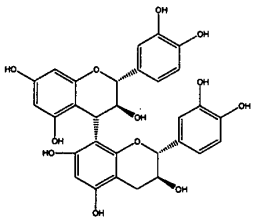
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gallocatechin



epicatechin



procyanidol

Primary pharmacodynamics

In Europe, the leaves of *Vitis vinifera* are documented in the literature of traditional medicine through several comprehensive reviews for their astringent and homeostatic properties where they are utilized in the treatment of diarrhoea, bleeding, haemorrhoids, varicose veins and other circulatory and venous diseases [Bombardelli 1997; Anonymous 2003; 2004; Petrini et al. 2003; Schneider, 2007i; 2007ii; Schneider et al. 2008; Schaefer & Red 2003; Volonté & Petrini 2004; Volonté et al. 2003; Weber, 2000].

Anti-inflammatory and anti-oedematous effect

In an *in vitro* study, venular endothelial cells were isolated from Wistar rats and cultivated on porous filters to confluent monolayers. These preparations respond to certain release products from simultaneously activated blood platelets and polymorphonuclear granulocytes (PMN) with a rise in hydraulic conductivity that, *in-situ*, would lead rapidly to local oedema, arteriolar constriction and venular thrombosis. In this model, selectively activated PMN alone induced only a modest increase in endothelial hydraulic conductivity that could be prevented by uric acid, an antioxidant. ASA prevented the activation of the blood cells. A standardized extract from grapevine leaves (AS 195), containing in particular the flavonoids quercetin-3-O-ß-D-glucuronide and isoquercitrin (quercetin-3O-ß-D-glucoside), not only prevented the deleterious effect of the release products on the venular endothelial monolayers but, applied promptly to an endothehium damaged by prior exposure to these release products, resulted in the repair of the endothehium [Nees et al. 2003]. In another study, the scavenge by procyanidines (polyphenol oligomers from *Vitis vinifera* seeds CAS 85594-37-2) of reactive oxygen species (ROS) involved in the onset and the maintenance af microvascular injury has been studied in phosphatidylcholine liposomes (PCL) using two different models of free radical generation. In an iron-promoted (Fenton-driven) model, procyanidines had a remarkable, dose-dependent antilipoperoxidant activity (IC50 = 2.5 µmol/l), more than one order of magnitude greater than that of the monomeric unit catechin (IC50 = 50 µmol/l). In the second model, procyanidines were highly effective in preventing conjugated diene formation in both the induction (IC50 = 0.1 µmol/l) and propagation (IC50 = 0.05 µmol/l) phases. The scavenging effect of tocopherol was weaker with IC50 of 1.5 and 1.25 µmol/l [Maffei Facino et al. 1994; Wollina et al. 2006].

The grapevine leaf extract is characterized by its content in flavonol glycosides and glucuronides, in particular quercetin-3-O-β-D-glucuronide, isoquercitrin (quercetin-3-O-β-glucoside), and kaempferol-3-O-glucoside. The most informative investigations were performed using confluent venular endothelial cells from animal and from human origin [Nees, 2003]. An attack by release products from simultaneously activated blood platelets (P) and polymorphonuclear granulocytes (PMN) leads to a breakdown of the venular endothelial barrier. Clinically this would result in formation of oedema and

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constriction of microvessels. Red vine leaf RVLE extract was able to support the repair of the venular barrier after an attack by said release products. These effects could be demonstrated on cells of from animal origin as well as on cells isolated from the human heart [Nees 2003i, 2003ii]. Quercetin-3-O-ß-D-glucuronide that had been isolated from the extract and was shown to be the major metabolite being present in human plasma after ingestion of RVLE acted in the same way. The extent to which cellular gaps opened and allowed the supernatant to flow through could be measured as "hydraulic conductivity". 0.7 mg dry RVLE/ml incubation medium nearly prevented the opening of the barrier in the presence of activated PMN/P. Preincubation of the venular cell layer with 50 µM quercetin-3-O-ß-D-glucuronide for 7 days markedly reduced the hydraulic conductivity compared to untreated cells.

In a publication by [Jonadet et al. 1983], the authors describe studies conducted with anthocyanosides extracted from *Vitis vinifera* (a), *Vaccinium myrtillus* (b) and *Pinus maritimus* (c). The results obtained *in vitro* indicated that these compounds inhibit elastase, a proteolytic enzyme which plays a role in the deterioration of conjunctive tissue and elastic fibers and is involved in certain pathological vascular conditions. The IC50 values are 0.13 mg/ml for (a), 0.20 mg/ml for (b) and 0.31 for (c). Lineweaver-Burk curves revealed that the inhibition was not competitive. Results obtained *in vivo* show that the angioprotective activities of these compounds can be classified in decreasing order as follows: (a), (b) and (c).
Nees developed a measurable and reproducible *in vitro* experimental model to investigate the effect of substances capable of modifying the hydraulic conductivity of the endothelial barrier of the venules. In *in vitro* experiments, this extract has been shown to have a "sealing" effect on the endothelium of the venules and a protective action against fluid extravasation induced by incubation with chemical mediators of inflammation [Nees S et al. 2003i].
Red vine leaf extract (RVLE) prevented the deleterious effect of the release products of P and PMN on venular endothelial cells. In addition, the extract was able to support the repair of the venular barrier after an attack by said release products [Smith, 1999].
The anti-inflammatory activity of the oligomeric stilbene a-viniferin from *Vitis* has been also tested as well as its mode of action though inhibition of cyclooxygenase-2 and inducible nitric oxide synthase [Chung et al. 2003].

Hepatoprotective activity

The hepatoprotective effect of ethanolic extract and its four different fractions ($CHCl_3$, EtOAc, n-BuOH, and remaining water fraction) of *Vitis vinifera* L. leaves was investigated against carbon tetrachloride (CCl_4)-induced acute hepatotoxicity in rats. The ethanolic extract was found active at 125 mg/kg dose (per os). The ethanolic extract was fractionated through successive solvent-solvent extractions and the n-BuOH fraction in 83 mg/kg dose possessed remarkable antioxidant and hepatoprotective activities. Liver damage was assessed by using biochemical parameters (plasma and liver tissue MDA [malondialdehyde], transaminase enzyme levels in plasma [AST-aspartate transaminase, ALT-alanine transferase] and liver GSH [glutathione] levels). Additionally, the pathological changes in liver were evaluated by histopathological studies. Legalon 70® Protect was used AS standard natural originated drug [Orhan et al. 2007].

Antimicrobial activity

Ethanol extracts of *Vitis vinifera* (leaves, raw fruits, young branches; 2:1:1, v/v/v), were investigated for their antimicrobial activity against 14 pathogenic bacterial species and a yeast, *Candida albicans*, using the agar well diffusion method, 19 Turkish traditionally used medicinal plants. *Vitis* leaves showed broad-spectrum antimicrobial activity [Oskay & Sari 2007].

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Antioxidative activity

The effect of grape leaf extract (GLEt) on antioxidant and lipid peroxidation states in liver and kidney alcohol induced toxicity has been evaluated. *In vitro* studies with DPPH* and ABTS* (cation radical) showed that GLEt possesses antioxidant activity. *In vivo* administration of ethanol (7.9 g/kg bw/day) for 45 days resulted an activity of liver marker enzymes (AST, ALT, ALP and GGT), lipid peroxidation markers (TBARS, lipid hydroperoxides) in liver and kidney with significantly lower activity of SOD, CAT, GPx, GST and non-enzymatic antioxidants (vitamin E, vitamin C and GSH) in liver and kidney AS compared with control rats. Administration of ethanol along with GLEt significantly decreased the activities of liver markers enzyme in serum towards near normal level. GLEt at a dose of 100 mg/kg was highly effective than 25 and 50 mg/kg body weight. In addition GLEt also significantly reduced the levels of lipid peroxidation and addition, significantly restored the enzymic and non-enzymatic antioxidants level in liver and kidney of alcohol administration rats. This observation was supplemented by histopathological examination in liver and kidney. These data suggest that GLEt exerts its protective effect by decreased the lipid peroxidation and improving antioxidants status, thus proving itself AS an effective antioxidant in alcohol induced oxidative damage in rats [Pari & Suresh 2008].
The antioxidative activity of the ethanolic extract of *Vitis vinifera* L. leaves was investigated. The ethanolic extract of *V. vinifera* leaves at 250 mg/kg dose was found to have the highest antioxidant activity [Sendogdu et al. 2006]. Comparable results have been published by [Kosar et al. 2007], from extracts from fresh, dried and brined leaves of *Vitis* [Monagas M et al. 2006].

Bronchodilatory activity

It has been investigated the effect of grape leaf hydroalcoholic extract on isolated rat tracheal contractions induced by KCl and acetylcholine. The trachea was removed from male adult Sprague-Dawley rat and placed in an organ bath containing Krebs-Henseleit solution and contractions were recorded isometrically. The results demonstrate that the grape leaf extract at 0.5, 1, 2, 4 and 8 mg/ml significantly reduces the tracheal contractions induced by KCl (60 mM) dose-dependently ($P<0.0001$). Acetylcholine (55 µM)-induced tracheal contractions were also attenuated at the same concentration of the extract ($P<0.0001$). The grape leaf extract induced relaxation in the KCl-induced contraction in trachea was unaffected neither by nitric oxide (NO) synthase inhibitor (L-NAME, 100 µM) nor by beta-adrenoceptor antagonist (propranolol 1 µM). Our results suggest that the bronchodilatory effect of grape leaf extract is mediated via the voltage dependent calcium channels on the smooth muscle cells membrane. Furthermore, the beta-adrenergic and NO are not involved. The extract was prepared from dried grape leaves 50 g were wixed with 230 ml of ethanol 70% for 72 hours at room temperature and stirred four times a day. The mixture was filtered and the solvent evaporated. The obtained extract was 9.5 g [Gharib Naseri & Heidari, 2006].

Vasorelaxant effect on isolated rat aorta

The relaxant effect of *Vitis vinifera* leaf hydroalcoholic extract (VLHE) on isolated rat thoracic aorta contractions induced by phenylephrine and KCl, and the role of aorta endothelium on this action has been investigated. Rat aorta was removed and placed in an organ bath containing Krebs-Henseleit solution and aorta contractions were recorded isometrically. The results demonstrate that the VLHE at 0.125, 0.25, 0.5, 1 and 2 mg/ml reduces the endothelial intact aorta contracted by phenylephrine (1 mu M) significantly and dose-dependently. Endothelial denuded aorta showed the same relaxation but in much less extent. The IC_{50} of these two groups were 0.454±0.08 and 1.73±0.23 mg/ml respectively. VLHE also reduced the aorta contractions induced by KCl (80mM). The relaxatory effect of VHLE on KCl-induced contractions were less than those evoked by phenylephrine. Soluble guanylate cyclase inhibitor (methylene blue, 10 mu M) and nitric oxide synthase inhibitor (L-NAME, 100 mu M) reduced the VHLE-induced relaxation in the intact aorta significantly but, atropine (1 mu M) was unable to decrease this vasorelaxant effect. These results suggest that the most vasorelaxant effect of VHLE

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on rat aorta is endothelium-dependent and also nitric oxide (NO) and cGMP are involved in this action [Gharib Naseri et al. 2004].

Spasmolytic effect

The effect of grape leaf hydroalcoholic extract (GLHE) on rat colon contractions induced by some spasmogens has been investigated. A piece of distal colon from male adult Wistar rats were dissected and mounted in an organ bath containing Tyrode solution and colon contractions recorded by an isotonic transducer under 1g resting tension. The GLHE (0.5- 4 mg/ml) reduced the contractions induced by KCl (60 mM), $BaCl_2$ (4 mM), acetylcholine (1 mu M) dose-dependently ($P<0.001$). The spasmolytic effect of GLHE on ACh-induced contraction was unaffected by propranolol (1 mu M), phentolamine (1 mu M), L-NAME (300 mu M), and naloxone (1 mu M). In Ca^{2+}-free but rich in KCl (120 mM) Tyrode solution, cumulative concentrations of $CaCl_2$ induced colon contractions which, were inhibited by the extract. Glibenclamide (3 mu M) had no effect on the extract spasmolytic activity, but tetraethylammonium (5 mM) contracted the pre-relaxed colon induced by the extract. Results suggest that the grape leaf hydroalcoholic extract spasmolytic effect is due to the blockade of the voltage dependent calcium channels and activation of Ca^{2+}-operated potassium channels [Gharib Naseri et al. 2006].
The effect of *Vitis vinifera* leaf hydroalcoholic extract (VLHE) on isolated rat tracheal contractions induced by KCl and acetylcholine has been also studied. The trachea was removed from male adult Sprague Dalwey rat and placed in an organ bath containing Krebs-Henseleit solution. The tracheal contractions were recorded isometrically under 1.5 g initial tension.

Results: The results demonstrate that the VLHE at 0.5, 1, 2, 4 and 8 mg/ml reduces the tracheal contractions induced by KCl (60 mM) significantly and dose-dependently ($P<0.0001$). Acetylchline (55 mu M)- induced tracheal contractions were also attenuated by the same extract doses significantly ($P<0.0001$). The VHLE-induced relaxation in the KCl-induced contraction in trachea was not affected neither by nitric oxide synthase inhibitor (L-NAME, 100 mu M) or beta-adrenoceptor antagonist (propranolol 1 mu M) and by muscarinic receptors antagonist (atropine 30 mu M).

Conclusion: These results suggest that the relaxant effect of VHLE on rat trachea is evoked via voltage dependent calcium channel blockage and beta-adrenoceptors, NO and cholinergic receptors are not involved in this relaxant effect of VHLE [Gharib Naseri & Heidari, 2006].

Diuretic activity

Aqueous and alcoholic extracts of *Vitis vinifera* leaves were tested for diuretic activity in rats. The parameters studied on individual rat were body weight before and after test period, total urine volume, urine concentration of Na^+, K^+ and Cl^-. In the present study alcoholic and aqueous extracts of *Vitis vinifera* leaves (100 mg/kg of body weight) showed increase in urine volume, cation and anion excretion. Furosemide was used as a reference diuretic [Shastry et al. 2002].

Secondary pharmacodynamics

Flavonoid-containing herbal preparations and isolated flavonoids have been reported to exhibit a wide range of biological effects, including antioxidant and enzyme-modulating actions and anti-allergic, anti-atherosclerotic, antithrombotic, antiviral, antibacterial, anti-inflammatory, antiproliferative, anticarcinogenic, antispasmodic and diuretic effects [Middleton et al. 1992, 1996; Hertog et al. 1996; Hollmann and Katan 1999; Pietta 2000].

In vitro tests
In the context with Chronic venous insufficiency (CVI) it would be of interest to evaluate whether grapevine leaf extract exhibits any effects on platelet aggregation. The effect of flavonoids on platelet aggregation was studied *in vitro* using platelet-rich plasma from four healthy male volunteers aged 24,

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29, 35 and 47 years. The flavonol glycoside quercetin 3-O-β-D-glucoside and various flavonoid aglycones including quercetin, apigenin and (+)-catechin were added to both platelet-rich plasma and washed platelets at concentrations of 0, 0.25, 2.5, 25, 250 and 2500 µmol/l. Indomethacin was used as a positive control. The results showed that the flavonoid aglycones inhibited platelet aggregation but that quercetin-3-O-β-D-glucoside did not significantly affect aggregation at any of the concentrations tested [Janssen et al. 1998].

In vivo tests
A randomised, placebo-controlled, multiple crossover study was conducted, in which 18 healthy volunteers (9 men and 9 women; mean age 25 ± 8 years; BMI 22 ± 1 kg/m^2) were given a preparation of onions equivalent to 114 ± 3 mg quercetin every day for 2 weeks in accordance with a fixed schedule. Blood samples were taken from the participants at defined intervals and the relationship between platelet aggregation and flavonoid concentration was plotted. The results showed that a daily dose of 114 mg quercetin did not have any significant effect on platelet aggregation. The mean plasma concentration of quercetin was 447 ± 117 ng/ml during the study period [Janssen et al. 1998].
Besides the flavonoids, grapevine leaves contain the following types of compound that are likely to be carried over to an aqueous extract [Bombardelli & Morrazoni 1995]:

- anthocyanins
- phenolic acids
- catechins and proanthocyanidins (flavanols)
- anorganic acids, sugars and mineral salts

The anthocyanins present in grapevine leaves are mainly glucosides of cyanidine, peonidine and malvidine [Boucheny A, Brum-Bousquet 1990]. Anthocyanins have been reported to possess antioxidant and vasoprotective properties. There are some doubts whether these effects measured in *in vitro* experiments or in animal studies using high doses are relevant for humans [Prior, 2004]. Absorption of anthocyanins appears to be low [Lapidot et al. 1998]. The amount of anthocyanins administered with 360 or 720 mg grapevine leaf extract is considered negligible in respect of the pharmacodynamic action of the substance.
The phenolic acids present in grapevine leaves are mainly derivatives of cinnamic acid, vanillic acid and caffeic acid [Boucheny A, Brum-Bousquet 1990]. These compounds are ubiquitous in vegetables and preparations of herbal origin and therefore not expected to contribute to the specific pharmacological effects of grapevine leaf extract.
The catechins found in grapevine leaves are mainly catechol and epicatechol [Boucheny A, Brum-Bousquet 1990]. Of the condensed catechins (proanthocyanidins) procyanidins B1 and B2 are the prevailing compounds, followed by procyanidins B3 and B4. Procyanidins have been reported to possess antioxidant and anti-oedematous activity [Bombardelli & Morrazoni 1995]. The study did not investigate the form in which the flavonoids were resent prior to administration. Onions are known to be rich in quercetin glucosides, especially quercetin-4´-glucoside and quercetin-3, 4´-O-diglucoside. After administration of fried onions quercetin glucuronides can be detected in plasma [Aziz et al. 1998; Graefe et al. 2001]. It can be supposed that quercetin glucuronides do not influence platelet aggregation *in vivo*.

Hypocholesterolemic effect

Oral administration of procyanidins from grape seed produced a hypocholesterolemic effect in a high-cholesterol animal feed model (rats). Specifically it prevented an increase in total and LDL plasma cholesterol and a decrease in HDL [Tebib, 1994].
In normal and hypercholesterolemic rabbit aortas, OPCs significantly lowered the amounts of cholesterol bound to aortic elastin compared to controls [Wegrowski, 1984].

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Antiproliferative activity

Procyanidin-rich fractions from grape *Vitis vinifera* extract showing different mean degrees of polymerization, percentage of galloylation (percentage of gallate esters) and reactive oxygen species-scavenging capacity were tested on HT29 human colon cancer cells. It has been observed that the most efficient fractions in inhibiting cell proliferation, arresting the cell cycle in G_2 phase and inducing apoptosis were the grape fractions with the highest percentage of galloylation and mean degree of polymerization. Additionally, the antiproliferative effects of grape fractions were consistent with their oxygen radical-scavenging capacity and their ability to trigger DNA condensation-fragmentation [Lizarraga et al. 2007].

Antidiabetic activity

The acute and subacute antidiabetic activities of the ethanolic extract of *Vitis vinifera* L., leaves were investigated. The acute effect was studied on the normoglycaemic, glucose hyperglycaemic and streptozotocin-induced diabetic rats; and the subacute effect was studied on same diabetic rats for 15 days. The blood glucose levels were measured by using blood glucose measuring strips based on glucose-oxidase method. The ethanolic extract of *V. vinifera* leaves at 250 mg/kg dose was found to possess a high antidiabetic activity. Mainly condensed tannins and flavonoids were suggested to contribute in the activity [Sendogdu et al. 2006].
The acute and subacute (15 days) hypoglycaemic and antihyperglycaemic effect of the two different doses (250, 500 mg/kg) of the aqueous extract from the leaves of *Vitis vinifera* L. were evaluated in this study. The aqueous extract was further fractionated through successive solvent extractions and the acute effect of different doses of its subfractions, 25 mg/kg for ethylacetate fraction, 80 mg/kg for *n*-butanol fraction and 375 mg/kg for remaining aqueous fraction were investigated using normal, glucose-hyperglycaemic and streptozotocin-induced diabetic rats. Blood glucose levels were measured according to the glucose oxidase method. Tolbutamide was used as a reference drug at a dose of 100 mg/kg. The antioxidant activity of the test samples was studied in the liver, kidney, heart tissues of diabetic rats by measuring malondialdehyde (MDA) and glutathion (GSH) levels. All results were compared to the diabetic control groups. The results showed that the EtOAc fraction was rich in polyphenolics and possessed a significant antihyperglycaemic and antioxidant activity equipotent with the reference hypoglycaemic agent (tolbutamide), when evaluated in diabetic rats [Orhan et al. 2006].

Other activities

The anti-herpes as well as the anti-mutagenic activities from the leaves and procyanidins from *Vitis vinifera* have been also assayed and published in the international literature [Girre et al. 1990; Liviero et al. 1993].

Pharmacodynamic interactions

Pharmacodynamic drug interactions of whole extracts or isolated constituents have not been reported. Various sources in the literature have suggested that the desired therapeutic effect of many flavonoids is usually obtained only after repeated oral intake of the respective preparations. This implies that the flavonoids and their metabolites accumulate in the body, and do not exert the pharmacodynamic effects seen in *in vitro* studies until a pharmacological threshold dose and relevant plasma levels has been reached. This hypothesis was confirmed by the results of a clinical study by [Kiesewetter et al. 2002]. A significant improvement in key CVI symptoms in the active treatment groups versus placebo could not be observed after 6 weeks, but became evident after 12 weeks of treatment. This could also explain why earlier studies reported a "small" or "undetectable" effect following single oral administration.

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3.1.1. Assessor's overall conclusions on pharmacology

The aqueous and ethanolic extracts of *Vitis viniferae* folium have been used as adjunctive therapy for Chronic venous insufficiency (CVI), and have shown a potential benefit [Abascal & Yarnell 2007]. Based on available preclinical data, it can be concluded that the mechanism of action of orally administered extract in CVI is not well known.

3.2. Overview of available pharmacokinetic data regarding the herbal substance(s), herbal preparation(s) and relevant constituents thereof

Data on the pharmacokinetics of the whole extract or relevant components are not available.

Flavonoids are the substances which are regarded as being responsible for the pharmacological properties of grapevine leaf extract. In the plant, flavonoids are generally present in genuine form as glycosides. Grapevine leaf extract as it has been referred previously contains as major flavonoid components quercetin-3-O-glucuronide, isoquercitrin (quercetin-3-O-glucoside) and kaempferol-3-O-glucoside. The substances present in blood after ingestion of plant extracts containing polyphenols usually differ from the native compound, as they are metabolites resulting from digestive and hepatic activity. The examination of pharmacokinetics and metabolism of the major and active constituents of such plant extracts in humans is therefore strongly recommended [Manach et al. 2005].
Various sources in the literature have suggested that the desired therapeutic effect of many flavonoids is usually obtained only after repeated oral intake of the respective preparations. This implies that the flavonoids and their metabolites accumulate in the body and do not exert the pharmacodynamic effects seen in *in vitro* studies until a pharmacological threshold dose and relevant plasma levels has been reached. This hypothesis was confirmed by the results of a clinical study by [Kiesewetter et al. 2000]. A significant improvement in key CVI symptoms in the active treatment groups versus placebo could not be observed after 6 weeks, but became evident after 12 weeks of treatment.

The grapevine leaf extract represents a complex mixture of different classes of compounds, having each their own pharmacokinetic characteristics. A significant portion of the components of grapevine leaf extract belongs to the group of polyphenols, such as anthocyanins, proanthocyanidins, catechins and phenolic acids [Bombardelli & Morazzoni 1995; Boucheny & Brum-Bousquet 1990]. Recently, [Manach et al. 2005] presented a review of various bioavailability studies in humans concerning polyphenols. They came to the conclusion that anthocyanins and proanthocyanidins are absorbed to a very low extent only. As an exception, the major circulating forms of anthocyanins are the intact glycosides [Manach et al. 2004]. The polymeric nature and therefore high molecular weight of proanthocyanidins should limit their absorption through the gut barrier. Hydroxycinnamic acids such as caffeic acid are rapidly absorbed from the small intestine and are conjugated, in particular glucuronidated in the same way as flavonoids. However these compounds are naturally esterified in plant products (as chlorogenic acid) and this impairs their absorption. Only the colonic microflora possesses esterases which are capable of hydrolyzing these bindings. Consequently, the efficacy of absorption of phenolic acids is markedly reduced for their esterified forms compared with the free form [Manach et al. 2004].

3.2.1. Assessor's overall conclusions on pharmacokinetics

No information is available on pharmacokinetic interactions. No information is available on metabolites.

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3.3. Overview of available toxicological data regarding the herbal substance(s)/herbal preparation(s) and constituents thereof

Single-dose toxicity

The toxicity of vine leaf aqueous extract (4-6:1) following a single oral dose was determined in SPF Wistar rats (3 males and 3 females per group). The doses used were 3000, 4000 and 10.000 mg/kg. There were no signs of toxicity and no mortalities over the 14-day observation period, and no pathological or histological changes were seen at autopsy.

The toxicity of vine leaf aqueous extract (4-6:1) following a single oral dose was also determined in CFI mice (3 males and 3 females per group). The doses used were 2000, 4000 and 10.000 mg/kg. There were no signs of toxicity and no mortalities over the 14-day observation period, and no pathological or histological changes were seen in autopsy [Rabe 2005; ESCOP, 2009].

Repeat-dose toxicity

The toxicity of vine leaf soft extract when given over a 90-day period was determined in SPF Mol-Wistar rats (12 males and 12 females per group) in a study which was subjected to QAU inspection and carried out in accordance with GLP standards. The animals were given oral doses of 25, 125 or 250 mg/kg/day, or a placebo. A satellite group given 250 mg/kg/day was subjected to follow-up observation. No mortalities occurred during the study period. Observations made during the study period on bodyweight, food intake etc. revealed no evidence of any systemic toxic effects due to the extract. The values found during haematological and clinical chemistry testing were within biological limits. The autopsy findings and the results of histological tests did not reveal any evidence of changes due to the extract. The doses of extract used were therefore judged to be non-toxic in the rat.

The maximum daily dose of extract is 720 mg, which represents a dose of 10.3 mg/kg for a person weighing 70 kg. This is about one-thousandth of the maximum dose of extract used in the single-dose toxicity studies. As the maximum dose used in these studies did not give rise to any signs of toxicity, it is extremely unlikely that any toxic effects will occur in man. Preparations containing vine leaf extract have been used in European Union countries for many years and there have been no reports of any harmful effects in man to date. On this basis, it is reasonable to assume that the products reviewed in this report will not pose any safety risk on therapeutic administration [ESCOP, 2009].

Genotoxicity

The mutagenic potential of vine leaf extract was assessed in a range of established *in vitro* and *in vivo* test models (Ames test, point mutation assay and chromosomal aberration assay). It was assessed in an Ames Salmonella/microsome plate assay using Salmonella typhimurium TA-100, TA-1535, TA-98, TA-1537 and TA-1538 with and without metabolic activation; S9 mix prepared from rat liver was used as the activation system. The assay was carried out in accordance with EU recommendations and OECD Guideline 471. Five concentrations of the vine leaf extract ranging from 8 to 5000 µg/plate were used, and positive and negative controls were also included. The results obtained did not provide any evidence to suggest that vine leaf extract has any mutagenic potential [ESCOP, 2009].

Mutagenic potential of grapevine leaf extract was also assessed in two independent point mutation assays carried out on the HGPRT locus of Chinese hamster V79 cells with and without metabolic activation; S9 mix prepared from rat liver was used as the activation system in each case. The assays were designed and conducted in accordance with the guidelines and recommendations on genotoxicity testing that were current at the time. On the basis of preliminary toxicity tests, a concentration range of 3.0 - 5000 µg/ml was selected for use in the assays. Positive EMS and DMBA controls as well as a

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vehicle control were included. Neither of the assays provided any evidence to suggest that the extract used had any mutagenic potential in the concentration range tested [ESCOP, 2009].

A micronucleus assay was carried out on NMRI mice (5 males and 5 females per group) to establish the potential of vine leaf soft extract for causing chromosomal aberrations. The assay, which complied with the relevant EU and OECD guidelines, was carried out using oral doses of the extract of 1.0, 3.0 and 10.0 ml/kg. Positive controls (cyclophosphamide 40 mg/kg) and vehicle controls (sodium chloride 0.9%) were included. Under the conditions employed, there was no significant increase in micronuclei frequencies in polychromatic erythrocytes.

The methanolic and aqueous extracts from Greek varieties of *Vitis vinifera* demonstrated *in vitro* antimutagenic effects against mutagenicity induced by bleomycin and hydrogen peroxide in Salmonella typhimurium strain TA102 [Stagos et al. 2006].
Oligomericproanthocyanidins from grape seed demonstrated in vitro antimutagenic activity [Liviero et al. 1993].
In addition, among the pharmacologically active flavonoids (quercetin, quercetin 3-O-β-D-glucuronide and isoquercitrin) in the vine leaf extract used is known that quercetin has given positive results in the Ames test. However, the mutagenic potential of quercetin has not been confirmed in *in vivo* tests carried out in rats and mice [Hertog & Hollman 1996].

Carcinogenicity

No data is available. However, it was demonstrated that anthocyanin-rich extracts from bilberry (*Vaccinium myrtillus* L.), chokeberry (*Aronia meloncarpa* E.), and grape *(Vitis vinifera*) inhibited multiple biomarkers of colon cancer in rats [Lala et al. 2006].
There has also been no evidence in the literature so far to indicate that flavonoids as a whole have any carcinogenic properties. Indeed, a large number of *in vitro* studies have been carried out which suggest that flavonoids have anticarcinogenic effects. However, the results obtained *in vitro* have yet to be confirmed in *in vivo* test models. Depending on the type and quantity of food consumed, the human intake of flavonoids is between 100 and 1000 mg per day [Middleton 1996].

In toxicity and carcinogenicity studies carried out on quercetin, promoted by the FDA, four groups of 50 male and female F344 rats were given 40, 400 or 1900 mg/kg of quercetin, or a placebo, in feed over a period of two years. There was no evidence of carcinogenic activity in the female rats and only slight evidence of carcinogenic activity in male rats. The carcinogenic effects, which took the form of renal tubule hyperplasia and nephropathy, were observed only in the high-dose group. No evidence of chronic toxicity was found [NTP: Toxicology and carcinogenesis studies of quercetin (CAS No. 117-39-5) in F344 rats (feedstudies) 1992].

Reproduction toxicity

The maternal and embryofoetal toxicity of an orally administered aqueous preparation of vine leaf soft extract was investigated in pregnant Himalayan rabbits in a study which was carried out in accordance with GLP standards and subjected to QAU inspection. Four groups of animals (12 per group) were used. One group received a placebo whilst the other three groups were given doses of 300, 1000 or 3000 mg/kg/day on days 6-18 of gestation. All doses were administered in a volume of 10 ml/kg. No signs of maternal toxicity were seen during the observation period and there was no visible evidence of foetal retardation. Slight, non-significant skeletal retardations and variations were seen in the foetuses of animals receiving the highest dose (3000 mg/kg of the extract per day). These variations were within biological limits in all cases and did not occur in any of the other groups. There was no evidence of changes due to the extract in any of the other findings, and no teratogenic effects were seen. On the basis of the results obtained, the NOEL for the extract was defined as > 3 g.

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In the reproduction study conducted with rabbits no evidence of any teratogenic effects was found. The recommended maximum dose is equivalent to a daily intake of 10.3 mg/kg of vine leaf extract. In the Leuschner study up to 3000 mg/kg/day of the extract, were applied without resulting in any teratogenic effect. The recommended maximum human dose of vine leaf extract is about 300 times lower than the highest dose used in the Leuschner study [Leuschner F, Mitterer KE, 1993]. Examination of the influence of *Extractum vitis viniferae* on the pregnant rabbit and the fetus by oral administration [LPT Report No. 7441/92. 1993; ESCOP, 2009].

Daily doses of 3000 mg/kg extract from grapevine leaves administered to female rabbits during organogenesis (6th to 18th day of pregnancy) did not exhibit teratogenic effects [Antistax® SPC, 2005].

3.3.1. Assessor's overall conclusions on toxicology

Several recent data on oral single-dose and repeat-dose toxicity, as well as on genotoxicity and reproduction toxicity of vine leaf have been reported.
In vitro and *in vivo* studies on the mutagenic potential of vine leaf extracts have not shown any evidence of mutagenic activity. Carcinogenic effects are not expected to occur, especially as vine leaf extract has been used in medicinal products for many years without any evidence of such effects being reported.

4. Clinical Data

4.1. Clinical Pharmacology

Pathophysiology of CVI

The term Chronic venous insufficiency (CVI) describes a clinical picture in which chronic venous diseases of diverse aetiology are manifested by similar or related symptoms and complaints. CVI is mostly caused by venous and capillary hypertension, which causes a persistent, chronic, venous stasis in the skin of the lower leg, manifested most characteristically upon postural challenge [Alguire & Mathes 1997]. The impairment of venous return that is responsible for the venous capillary hypertension can be caused by degenerative, dilating venous conditions of the superficial (primary varices), the transfascial (insufficiency of the perforating veins), or the deep system ("deep" varicosis, insufficiency of the main veins) [Jünger et al. 1994]. Chronically disturbed haemodynamics of deep or superficial veins due to obstructed venous segments or valvular incompetence lead usually to trophic changes in the inner ankle area of the lower limbs and disturbances in the microcirculation of the skin have been considered to be major contributors for skin changes associated with chronic venous hypervolaemia and venous hypertension. Cutaneous microangiopathy of clinical relevance such as enlarged, tortuous capillaries surrounded by micro-oedema contributes to the skin alterations in the lower limbs and determines the course of CVI [Fagrell 1995; Jünger et al. 1996]. CVI can be classified according to its haemodynamic, morphological, and clinical aspects. The clinical grading system, based on Widmer's classification, as well as other international classification systems, e.g. CEAP (Clinical Class, Etiology, Anatomy and Pathophysiology), [Widmer LK, 1981; Partsch H, 1994; Porter JM et al. 1995]. The most important therapeutic approaches include compression therapy, physiotherapy, surgical treatment and sclerotherapy.

Drug therapy

Drug therapy is a treatment option in incipient and mild to moderate Chronic venous insufficiency (CVI) (Widmer grades I and II, CEAP Clinical classes 2, 3, or 4a).

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Topical drug treatment

Antioedema drugs are mostly plant derived substances for which an antiexudative and antioedematous action and efficacy have been confirmed in experimental studies and clinical trials. Experimentally, most of them have a membrane stabilising action that leads to a decrease in capillary permeability. Some of these substances also have venotonic properties.
Long-term treatment with antioedema drugs can also be considered in primary and secondary CVI or if invasive measures are not applicable [Gallenkemper et al. 1998]. The majority of these products contain plant extracts or semisynthetic derivatives of plant-derived substances. The most commonly used are coumarins (α-benzopyrone derivatives); saponins/horse chestnut seeds extracts; flavonoids (γ-benzopyrone derivatives); rutin, diosmin, and hesperidin; anthocyanins and procyanidins.
Procyanidins, like the anthocyanins, are flavonoids that are not available as monosubstances. Procyanidins are present in relevant amounts in standardised extracts such as grapevine leaf extracts (*Vitis vinifera*) and are formed through condensation reactions of flavonols [Hostettmann K et al. 1994].
The complex syndrome of symptoms involved in CVI led to investigate the activity of vine leaf preparations on two distinct parameters affecting CVI. On the one hand the effects of the product on microcirculation, on the other on the typical objective and subjective symptoms of CVI such as the presence of oedema and the typical CVI complaints "tired, heavy, and swollen legs", or tension and pain in the legs.

4.1.1. Overview of pharmacodynamic data regarding the herbal substance(s)/preparation(s) including data on relevant constituents

No data available.

4.1.1.1. Assessor's overall conclusions on pharmacodynamics

At present, the mechanism of action of vine leaf water extract in chronic venous insufficiency cannot be considered clarified.

4.1.2. Overview of pharmacokinetic data regarding the herbal substance(s)/preparation(s) including data on relevant constituents

The vine leaf extract is a complex extract containing more than 200 different identified substances. The clinical effects of the grapevine leaf extract cannot be attributed to a specific individual constituent but can be ascribed with reasonable plausibility to the extract as a whole. It is therefore virtually impossible to perform classical pharmacokinetic studies with the complete product and pharmacokinetic studies of individual constituents such as flavonoids will only provide limited information with regard to clinical efficacy.

Flavonoids (quercetin glucuronide, kaempferol glucosides) are quantitatively important constituents of the whole extract. The systemic bioavailability of flavonoids is probably relatively low and variable. Orally administered flavonoids are susceptible to pre-systemic metabolism by the intestinal flora. It is likely that the absorbed metabolites formed by the microflora in the gut contribute to the biological action of orally administered flavonoids. Systemic flavonoids are metabolised in the liver primarily by conjugation (methylation or sulfuronidation) and/or glucuronidation. The resulting metabolites are excreted either with the urine or with the bile into the intestine, where they undergo further metabolism. The pharmacokinetic input function of systemic flavonoids may well be further extended by this enterohepatic recycling process [Manach & Donovan 2004].

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4.1.2.1. Assessor's overall conclusions on pharmacokinetics

Data on pharmacokinetics of vine leaf extract or relevant components are not available in humans. No reliable methods exist to determine simultaneously the plasma levels of all active ingredients present in the whole extract.

4.2. Clinical Efficacy

Several publications referring to human clinical studies with medicinal products containing *Vitis vinifera* were found in the literature.
Three of the published studies investigated the safety and efficacy of a dry extract of grapevine leaves (4-6:1) extraction solvent water (AS 195).
The safety and efficacy specific information of these studies is presented below.

The clinical information available for aqueous extracts of grape vine leaf investigates the activity on two distinct parameters affecting Chronic venous insufficiency (CVI). On the one hand the effects of the product on microcirculation, on the other on the typical objective and subjective symptoms of CVI such as the presence of oedema and the typical CVI complaints "tired, heavy, and swollen legs", or tension and pain in the legs.

Clinical parameters measured

In all clinical studies, changes in leg volume and/or calf and ankle circumference were included in the endpoints studied. All confirmatory studies planned to show efficacy on subjective symptoms of CVI used the lower leg volume determined by water displacement plethysmography as the primary efficacy endpoint. Other endpoints included the lower leg diameter (at calf height and at the ankle), visual analogue scales (VAS: 0-10 cm) for the main symptoms ("tired, heavy legs", "sensation of tension", "prickling sensation", and "pain in the legs"), and the global assessment of efficacy and tolerability by the patient and by the doctor according to a 4-point verbal rating scale (VRS: "good", "satisfactory", "not satisfactory", and "poor"). These measurements are consistent with the guidelines of the German Society for Phlebology.
In the double-blind, placebo-controlled cross-over study with two treatment sequences of 6 weeks each separated by a 4-week placebo washout period, microcirculation in the most affected CVI leg was measured by laser doppler fluxmetry (685 nm, penetration depth approximately 1 mm) and transcutaneous oxygen monitoring.
Patients were usually questioned about their subjective well-being. They were also instructed to spontaneously report any adverse events experienced. Laboratory tests (haematology, clinical chemistry, and urinalysis) and blood pressure and pulse measurements were carried out in most studies to record safety.

4.2.1. Dose response studies

From the results of [Kiesewetter et al. 2000], a single daily dose of 360 mg of grapevine leaf extract is as effective and well tolerated as a daily dose of 720 mg. On average, the higher dose regimen offers greater efficacy in terms both of the extent and duration of action, but is roughly equivalent with regard to subjective symptoms reduction. The lower dose, however, is considered to be sufficient in the majority of patients and has been chosen to reduce the intake of extract, thus most likely decreasing the likelihood of observing adverse events. Consequently, this is the dosage that has been approved in some marketing authorisations.
No pharmacokinetic or pharmacodynamic studies, however, were performed to support the posology and daily dose proposed.

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4.2.2. Clinical studies (case studies and clinical trials)

Several publications referring to human clinical studies investigating the safety and efficacy of a dry extract of red *Vitis vinifera* leaves (4-6:1) extraction solvent water (AS 195) were found in the literature. Some others studies have been assessed in this AR, that originate from confidential in house files of Boehringer Ingelheim. The company allowed using those data for assessment and monograph/list entry establishment.

Double-blind, Placebo-controlled studies

[Kiesewetter et al. 2000]: In a 12-week, randomized, double-blind, placebo-controlled, parallel-group, multicenter study conducted in accordance with the principles of Good Clinical Practice (GCP), the efficacy and safety of once daily doses of 360 and 720 mg RVLE (AS 195) were compared to placebo in male and female outpatients aged between 21 and 80 years old (with 52% ≥ 60 years) with body weights ranging from 46 to 120 kg (Broca index: median 7.3, range: -30 to +82%; this correspond to a BMI of approx. 25 kg/m^2). And stage I and incipient stage II Chronic Venous Insufficiency (CVI) with no major dystrophy of the skin. The majority (72%) of the patients were women. Compression therapy and the administration of diuretics were not permitted during the study period.
Patients were randomly assigned to a double-blind treatment with placebo, 360 mg RVLE or 720 mg RVLE once daily for 12 weeks, preceded and followed by a single-blind 2-week placebo treatment for baseline run-in and end-of-trial washout, respectively. Study criteria were evaluated at baseline, after 6 and 12 weeks of treatment and 2 weeks after discontinuation of treatment. Of the **260** patients enrolled and randomized, 219 completed the study in accordance with the protocol, 86 test subjects took a single daily dose of 360 mg AS 195, 84 took 720 mg AS 195/day and the remaining 87 test subjects received a placebo. During weeks 13 and 14 none of the participants received any further medication.

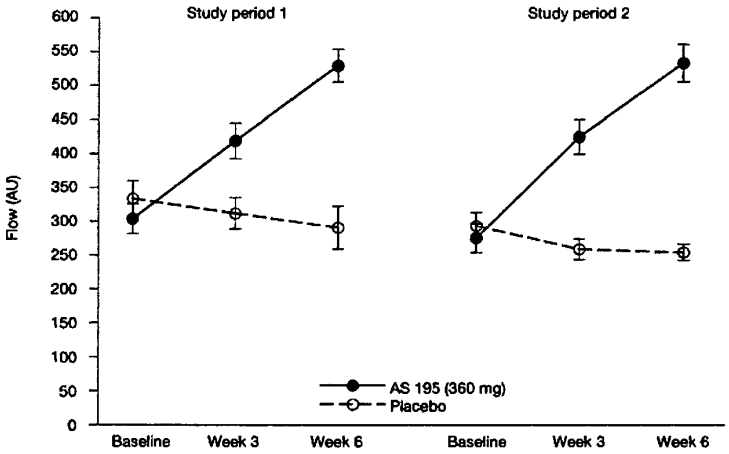
Subjectively, there was an improvement in CVI symptoms at 6 weeks with all treatments, but a further improvement at week 12 was seen only in the active treatment groups: at 12 weeks, the changes compared to baseline were significantly greater ($p < 0.001$) in both active treatment groups than in the placebo group. The study demonstrated that once daily doses of 360 and 720 mg RVLE were effective in the treatment of mild CVI, reducing significantly lower leg edema and improved CVI-related symptoms to a clinically relevant extent.

The treatments were well tolerated. All 260 patients who were enrolled and randomized were included in the safety analysis. This included three patients who withdrew from the study before visit 2 (i.e. before entering the treatment phase). 257 patients received at least one dose of the randomized study medication. Most patients completed the study up to 84 days of treatment. The mean duration of treatment for the 360-mg dose of AS 195 was 81.9 days (n = 86) whilst the mean duration of treatment for the 720-mg dose of AS 195 was 79.7 days (n = 84). Placebo was taken by 87 patients during the randomized treatment period (mean duration of treatment: 79.3 days) and by all patients during the 14-day run-in period (n = 260) and the 14-day follow-up period (n = 246). A total of 34 adverse events (AEs) were reported in 31/260 patients: 3 AEs in 3/260 subjects treated with placebo during the run-in phase; 3 AEs in 3/246 patients treated with placebo during the follow-up period; 10 AEs in 10/87 patients treated with 360 mg AS 195; 2 AEs in 2/85 patients treated with 720 mg AS 195; and 16 AEs in 13/88 patients treated with placebo. Most AEs were considered to be mild (n = 21) or moderate (n = 9). Four AEs were classified as severe, three in patients taking the placebo (phlebitis of the large saphenous vein; knee surgery; abdominal discomfort) and one in a patient taking 360 mg AS 195 (leg hematoma following minor trauma). Six AEs were rated as likely to be causally related to the study medication by the investigator; of these, two (mild constipation and mild hair thinning) were

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reported in patients treated with 360 mg AS 195, whilst the other four occurred in patients treated with placebo. Gastrointestinal disorders (abdominal discomfort, diarrhea, dyspepsia, dry mouth or retching) were the most frequently cited AEs (n = 11), followed by infections (n = 6), headache (n = 4) and disorders of the musculoskeletal system (n = 4). Two AEs which occurred during treatment with placebo required hospitalization and were labelled as „serious"; there was one incident of vaginal hemorrhage during the run-in phase and one incident of severe acute osteoarthritic complaints in the knee of the affected leg during the randomized treatment period. Three other patients were withdrawn following an AE: 1 patient was withdrawn during the placebo run-in period due to moderate abdominal discomfort; 2 patients were withdrawn from randomized treatment with the placebo, one because of phlebitis of the greater saphenous vein and one because of severe abdominal discomfort. The clinical laboratory data, blood pressure and pulse rate data and 12-lead ECG recordings did not indicate any significant mean or individual changes which were likely to have been due to the study medications. Overall tolerability was rated as follows for the 3 treatments: 360 mg AS 195: good 81%, satisfactory 19%; 720 mg AS 195: good 94%, satisfactory 6%; placebo: good 68%, satisfactory 30%, not satisfactory 2%. The study was conducted in multicentric settings, but most measurements were carried out by only two investigators, thus this trial can be considered basically a monocentric one

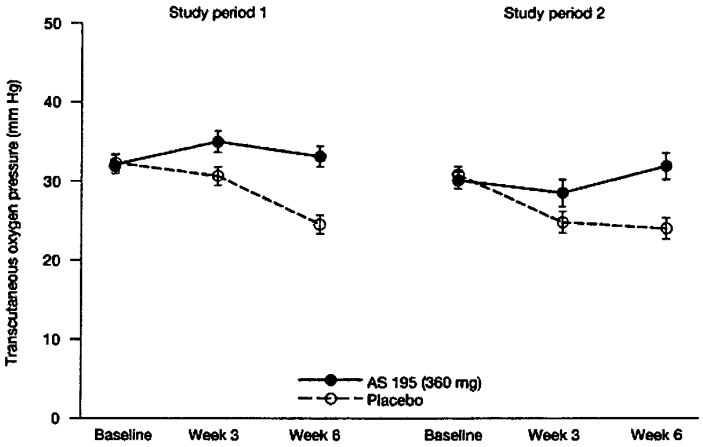
[Kalus et al. 2004]: The effect of AS 195 on cutaneous microvascular blood flow, transcutaneous oxygen pressure (tcpO2), and leg oedema was investigated in a randomised, double-blind, placebo-controlled, crossover study in male and female patients, aged ≥ 18 years with an average age of 66 years, with confirmed CVI stage I or II. In this efficacy study, **71** patients (70 during the second phase) were treated with either placebo or 360 mg AS 195 film coated tablets. The first group (n=36) received AS 195 360mg once daily during a first 6-week treatment period, followed by a 4-week placebo washout period and then placebo during the second 6week treatment period. The second group (n = 35) started with placebo and received AS 195 360mg after the placebo wash-out. After 6 weeks, patients in the AS 195 group had increased microvascular blood flow values and transcutaneous oxygen pressure (tcpO2). The active substance group showed a statistically significant increase of 241.8±18.7 Arbitrary Units (AU), while a reduction of 41.0±18.7 AU was recorded in the placebo group ($p<0.0001$).



Microcirculation, measured in arbitrary units (AU) (laser Doppler flow measurement at Doppler frequencies of 10.1–37.2 kHz, mean ± SEM, after 10 minutes of standing) (from [Kalus et al. 2004])

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The $TcpO_2$ oxygen reading also rose significantly from baseline levels in the AS 195 group, by 1.35±0.97 mmHg, contrasting with a reduction in the placebo group of 7.27±0.97 mmHg.



Transcutaneous oxygen pressure (mean ± SEM, after 10 minutes of standing) (from [Kalus et al. 2004])

These changes in the measured parameters were associated with corresponding volume changes in the lower leg. After just three weeks of treatment, statistically significant differences were also observed in ankle and calf circumference. After six weeks, these circumferences had fallen by 0.39 and 0.54 cm respectively, in the active group, compared to increases in the placebo group of 0.29 and 0.14 cm respectively. The authors of the study concluded that the administration of AS 195 could have a positive effect on the course of CVI. The minimum number of days of exposure was 38 in the AS 195 and 24 in the placebo group, the maximum number 46 in the AS 195 and 45 in the placebo group. Thirteen (18.3%) subjects out of 71 experienced 16 adverse events (AEs) during the 16 week trial. Ten AEs (14.1%) were of mild, 4 (5.6%) of moderate and 1 (1.4%) of severe intensity. None of the AEs were assessed as related to the trial medication. One patient died from a cardiac arrest. He had been treated with placebo and never received AS 195 in this trial. All patients assessed the overall tolerability as good or satisfactory. Laboratory parameters did not change during the study [Kalus et al. 2004].

[Limoni C, 1996]: A four-week, multicentre, randomised, double-blind versus placebo, parallel-group safety trial to evaluate the tolerability profile of certain capsules (water extr. *Vitis vinifera* siccum 4-6:1) in male and female patients suffering from chronic venous insufficiency. The study population consisted of **105** patients, male and female, aged between 20 and 70 years with chronic venous insufficiency of grade I, II, and III according to Widmer. The patients received 3 capsules of that extract or placebo per day during 28 days. Seventy-two patients were randomised into the active treatment group and 33 into the placebo treatment group. In the absence of a strictly diagnosed CVI, patients with a subjective feeling of heavy and tired legs were admitted as well. Primary endpoints were the incidence of adverse events and the rating of the overall tolerability. Secondary endpoints were laboratory tests and physical examination. There were no differences in tolerability between the two treatment groups. The proportion of volunteers reporting adverse events was similar in both treatment groups: 40.2% in the active treatment group and 36.4% in the placebo group. The most frequent adverse events were mild gastrointestinal disorders, sleep disturbances, and tiredness. Laboratory values did not change in either treatment group during the trial period. The capsules were well tolerated by 84.9% of the volunteers. No severe adverse events were reported.

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[Vix et al. 2007]: This 12-week, double-blind, randomised, placebo-controlled, multicentre trial was carried out to evaluate the efficacy and tolerability of film-coated tablets, extr. *Vitis vinifera* (***4-6:1 water***) 360 mg/day orally, in male and female patients suffering from chronic venous insufficiency. The design and methodology used closely reflected those used in BI trial by Schaefer 2004).
The time course of the change from baseline in limb volume was similar for both treatment groups with a more marked improvement of leg oedema over time in patients treated with these tablets. After 84 days of treatment, limb volume was reduced by -21.38 (±11.30) g of displaced water in the verum group. The adjusted mean reduction in limb volume in the placebo group was less pronounced with -10.71 (±11.56) g. However, the difference between treatment groups (-10.67 ±14.35 g) was not statistically significant.

For the secondary endpoint "change from baseline in calf circumference", the adjusted mean difference to placebo after 42 days of treatment with these tablets was -0.42±0.22 cm in the FAS (p=0.0596) vs. -0.31±0.24 cm on study day 84 (p=0.1851). In the Per-Protocol Set (PPS), the difference to placebo was -0.47 (±0.23) cm on study day 42, with a p-value just reaching significance at 0.0445. The evaluation of the four subjective symptoms of CVI, as assessed by VAS, yielded similar results: after 42 days of treatment, the adjusted mean differences between active treatment and placebo were -0.44 cm for "tired, heavy legs", -0.73 cm for "sensation of tension in the legs", and -0.48 cm for "pain in the legs" (vs. -0.38 cm, -0.04 cm, and 0.18 cm on study day 84). There was no difference between treatment groups regarding the symptom "tingling sensation in the legs". Global efficacy was rated as good or satisfactory by 70.9% of all patients treated with these tablets and 67.7% of all patients allocated to placebo whereas the investigators' ratings were 69.0% and 62.6%, respectively. The assessment of quality of life by the Tuebingen QoL questionnaire resulted in a better mean score for verum group compared with placebo regarding the subscale "leg complaints", the subscale most specifically related to CVI. The differences, however, were not statistically significant.

[Schaefer & Petrini 2004]: This placebo controlled, randomised, parallel group study was carried out in 2004. **247** CVI patients (CEAP-Scores 3 or 4a) were entered in the study: 121 were treated with the grapevine leaf extract AS 195 360 mg as a single tablet and 126 with placebo for 12 weeks. For the primary endpoint, the difference related to placebo, adjusted for centre effect and baseline values, in changes of displaced water from baseline until Day 84 was -10.42 cm (SE: 11.84; 95% CI: -33.76, 12.92). The difference in changes between the two treatment groups was not statistically significant. Subjective CVI symptoms diminished in both treatment arms similarly during the first three weeks. A statistically significant difference between the two treatment groups in favour of verum, was found for the symptoms "sensation of tension in the legs" at Day 42 (p=0.0211, ANCOVA) and "pain in the legs " at Day 84 (p=0.0222, ANCOVA). At all other time points no statistically significant difference was detected between the two treatment regimens for the symptoms "tired heavy legs" as well as "tingling sensation" in the legs. After 84 days of treatment, the limb volume as measured by the water displacement method was statistically not significantly more reduced in subjects allocated to verum than in those randomised to placebo. Careful inspection of the results showed that the unexpected high intra- as well as inter-individual variability of limb volume determinations contributed significantly to the outcome of the study. The variability between 2 or 3 replicates of the limb volume determination, for example, exceeded 20 g of displaced water in more than 20% of patients (range 0% to 44.1%) in 8 out of 23 study sites (35%). This study was negative in terms of a primary endpoint, although there was evidence of symptomatic benefit from the active treatment.

[Schaefer E & Petrini LE, 2003]: A 17 week, randomised, double-blind, placebo controlled cross-over trial to evaluate the efficacy of water extr. *Vitis vinifera* ***4-6:1*** film coated tablets (AS 195) given as 2 capsules of 180 mg of AS 195 daily 360 mg/day p.o., in **179** male and female Japanese patients with swellings of calf and ankle due to disorders of venous reflux belonging to Class 1 according to Porter's classification, in improving microcirculation of the skin in the leg of male and female patients

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suffering from chronic venous insufficiency The microcirculation study has demonstrated that objective signs associated with CVI (microvascular blood flow, $tcpO_2$, ankle circumference and calf circumference) can be improved significantly after just 3 weeks of oral treatment with a single daily dose of 360mg of the grapevine leaf extract AS 195. These results build on those from Harrison trial (1998) showed that AS 195 reduces lower leg oedema and calf circumference in patients suffering from CVI treated once daily for 12 weeks. Out of the 179 evaluable subjects 38.5% experienced a marked and 42.5% a moderate global improvement of CVI symptoms. Improvement was rated as "moderate" by 59.8% of the patients with regards to heaviness/tiredness, 69.9% with regards to tension, 79.2% to tingling, 74.0% to pain, and 74.4% to itching. Circumference of calf (mean±SD) was 33.5±2.99 cm prior to treatment and 33.0±2.94 cm at 12 weeks, of the ankle (mean±SD) 22.3±1.84 cm prior to treatment and 21.9±1.82 cm at 12 weeks after treatment ($p<0.05$). These differences were not statistically significantly different from baseline.

Open-label, Observational studies

[Schaefer et al. 2003]: The 6-week, open, uncontrolled multicentre safety trial was conducted to specifically evaluate the tolerability and safety of AS195 film-coated tablets, 360 mg/day *per os*, in **65** male and female patients, aged 25 - 82 years, suffering from chronic venous insufficiency grade I or II (Widmer classification). The verum group received two film-coated tablets once daily for 42 days (360 mg/day). The primary objective of the study was to assess the tolerability and safety of AS 195 extr. *Vitis vinifera* siccum ***4-6:1***) film-coated tablets. The observational study was conducted in accordance with the requirements of the Swiss licensing authority (Swissmedic: application document) in a total of 11 study centres (general practitioners) between June and October 2002. Three visits (study enrolment, baseline examination at the start of the study, final examination at the end of the study, with a follow-up examination if necessary) were planned. The study was carried out in accordance with GCP. At the end of the study, all subjective symptoms of CVI (tired, heavy legs, sensation of tension in the legs, tingling sensations in the legs, pain in the legs) were statistically significantly improved. The global assessment of efficacy by the patients and by the investigators was rated as good or satisfactory in most of the patients. The primary variable was the number and intensity of adverse events (AEs), with particular emphasis placed on AEs regarded by the investigator to be treatment-related. Secondary safety variables included the global assessment of tolerability by both the patients and investigators as well as vital sign assessments. AS 195 film coated tablets were well tolerated during the whole study. Overall, six patients (9.2%) experienced a total of 7 AEs of mild or moderate intensity judged by the investigator as potentially causally related to study medication. They included gastrointestinal problems (4 patients), headache (1 patient), anorexia (1 patient), and erythematous rash (1 patient). Four patients of whom experienced a significant AE necessitated study discontinuation in three cases and dose reduction in one case. Most patients rated the global tolerability of AS 195 film-coated tablets as good (49 patients, 75.4%) or satisfactory (12 patients, 18.5%). Three patients (4.6%) rated tolerability as bad. One patient (1.5%) gave no rating. The global assessment of tolerability by the investigators was in good agreement with those of the patients. The investigators rated the tolerability as good in 50 patients (76.9%), as satisfactory in 10 patients (15.4%), as not satisfactory in one patient (1.5%), and as bad in two patients (3.1%). No relevant changes of vital signs (systolic and diastolic blood pressure, pulse rate) were measured. Patient´s compliance was rated as good [Schaefer et al. 2003].

Assessment The present open-label, multicentre observational study demonstrated that AS 195 film-coated tablets were effective and well tolerated in outpatients with chronic venous insufficiency grade I or II supervised in a setting of private practices. No change of the safety profile has been noted.

[Monsieur & Van Snick 2006]: A small, open observational clinical study was conducted in **39** patients suffering from Chronic Venous Insufficiency (CVI), grade I or II according to the Widmer

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classification, (grade 2 to 4 of the international CEAP classification). Patients were treated with 180 mg of RVLE (AS 195) twice daily (360 mg in total) for six weeks. The parameters investigated were objective measurements of lower leg volume and the circumference of the leg as well as subjective criteria such as heaviness and pain in the leg. A clear and significant improvement of all parameters was observed after two weeks of treatment. This effect was still present and increased slightly after four and six weeks in all objective and subjective parameters tested in this study.

Assessment This study has shown a fast onset of action and an efficacy of the AS 195 extract in chronic venous insufficiency. Adverse events/side effects: not specified. In general, the treatments were well tolerated. Three patients prematurely stopped the trial for reasons not linked to the treatment or to the disease. No change of the safety profile.

Clinical studies with grapevine leaf extract (4-6:1, extract solvent: water) (AS 195)

Study ID	**Study Dates** **Persons**	**Design Control type Study objective**	**Study &, Ctrl Drugs Dose, Route, & Regimen**	**Subjects per Arm treated / completed**	**Duration**	**Gender M/F Median Age (Range)**	**Diagnosis Inclusion Criteria**	**Primary Endpoint**	**Outcome**
Kiesewetter, 2000	April Sept-1998 **260**	randomized, double-blind, parallel, dose response Placebo Efficacy, tolerability, safety	AS 195 caps 180mg, *per os* 2-0-0 AS 195 capsules 360 mg, *per os* 2-0-0 Placebo	360 mg : 86/84, 720 mg :84/79 87/80	12 wks	24/62 56.2±12.4 years (mean±SD) 27/57 55.7±13.8 years (mean±SD) 20/67 59.2±11.5 years (mean±SD)	CVI Grade I, II according to Widmer	Baseline adjusted changes of lower limb volume assessed by water displacement plethysmography	Positive
Schaefer & Petrini 2004	2004 **247**	randomized, double-blind, parallel Placebo Efficacy, tolerability	AS 195 tablets 360 mg, *per os* 1-0-0 Placebo 1-0-0	360 mg : 121/117 126/123	12 wks, 2 wks single blind placebo	20/99 53.0 years (20–83 years) 15/111 53.0 years (23–79 years)	CVI CEAP-Scores 3 or 4a	Baseline adjusted changes of lower limb volume assessed by water displacement plethysmography	Negative
Schaefer & Petrini 2003	2003 **179**	randomized, double-blind, controlled cross over trial Placebo Efficacy	AS 195 tablets 360 mg, *per os* 1-0-0 Placebo 1-0-0	360 mg	17 weeks	No data	CVI Grade I, II according to Porter	Baseline adjusted changes of lower limb volume Changes in CVI symptoms	Negative
Vix et al. 2006	2006 **211**	randomized, double-blind, parallel Placebo Efficacy, tolerability	AS 195 tablets 360 mg, *per os* 1-0-0 Placebo 1-0-0	360 mg : 103/98 99/95	12 wks	32/71 61.0 years (29–86 years) 26/73 61.0 years (30–87 years)	CVI CEAP Scores 3 or 4a	Baseline adjusted changes of lower limb volume assessed by water displacement plethysmo-graphy	Negative
Kalus 2004	2002, completed **71**	randomized, double-blind, cross-over Placebo	AS 195 tablets 360 mg, *per os* 1-0-0	360 mg : 70/70	6 wks	16/55 65.2±7.7 years (mean±SD) (32–76)	CVI Grade I, II according to Widmer	resting flux, change from baseline measured by Laser Doppler fluxmetry	Increase of microvascular blood flow

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Study ID	Study Dates Persons	Design Control type Study objective	Study &, Ctrl Drugs Dose, Route, & Regimen	Subjects per Arm treated / completed	Duration	Gender M/F Median Age (Range)	Diagnosis Inclusion Criteria	Primary Endpoint	Outcome
		Efficacy, tolerability	Placebo 1-0-0						
Schaefer et al. 2003	June 2001 Oct-2001 **65**	Open/ Observational Uncontrolled Tolerability, safety, Efficacy,	AS 195 tablets 180 mg, *per os* 2-0-0	360 mg : 65/59	6 wks	7/58 M: 65.9 years (mean) (56-82 years) F: 56.6 years (mean) (25–80 years)	CVI Grade I, II according to Widmer	Incidence and Intensity of adverse events; Changes in CVI symptoms as secondary endpoint	Safety study Positive
Monsieur 2006	2006 **39**	Open Tolerability, safety, Efficacy	AS 195 tablets 180 mg, *per os* 2-0-0	360 mg	6 wks		CVI Grade I, II according to Widmer	Baseline adjusted changes of lower limb volume Changes in CVI symptoms	Positive
Limoni 1996	Dec-1996 May 1997 **105**	randomized, double-blind, parallel, dose response Placebo Multicentre Tolerability, Safety	AS 195 caps. 180 mg, *per os* 1-0-2 Placebo 1-0-2	540 mg : 72/64 33/27	4 wks	6/66 45.2 years (18.4–67.9 years) 4/29 36.7 years (25.6–70.6 years)	CVI Grade I, II. III according to Widmer	Safety Global improvement	Safety

4.2.3. Clinical studies in special populations (e.g. elderly and children)

No information available.

4.3. Overall conclusions on clinical pharmacology and efficacy

The clinical efficacy of an extract from red vine leaves (RVLE; AS 195) was investigated in double blind, placebo-controlled studies [Kiesewetter et al. 2000; Kalus et al. 2004] and in open observational studies [Schaefer et al. 2003; Monsieur & Van Snick, 2006] during treatment of chronic venous insufficiency. In these studies, the RVLE was showed an acceptable clinical efficacy and tolerability, while there were some others with negative results [Schaefer & Petrini 2003; 2004; Vix et al. 2006]. Treatment with RVLE has been shown to lead to a reduction of leg volume, measured using both water plethysmography and calf and ankle circumference, and of the subjective symptoms investigated in the studies reviewed.
The extract AS 195 was consistently more effective than placebo in reducing leg oedema and improving symptoms of Chronic Venous Insufficiency (CVI) across most efficacy parameters evaluated, even though not always a statistically significant superiority could be shown.

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5. Clinical Safety/Pharmacovigilance

5.1. Overview of toxicological/safety data from clinical trials in humans

The safety profile of vine leaf extracts can be described as acceptable from all clinical studies in Chronic Venous Insufficiency (CVI) patients and from its use from products on the market. The safety results obtained from the clinical studies conducted so far show that the oral use of vine leaf extracts are well tolerated by most patients. No drug-related serious adverse events were reported during the clinical trials, and when reported that were mild and transient. Seven non-serious adverse events (constipation, dermatitis, headache, hair thinning, menometrorrhagia, urticaria) or moderate (erythematous rash) of mild intensity leading to patient withdrawals, were suspected to be related to the active trial medication.

5.2. Patient exposure

The controlled studies evaluated 1073 patients in total [Kiesewetter et al. 2000; Kalus et al. 2004; Schaefer & Petrini 2004; Vix 2006, Limoni 1996]. The open studies, evaluated by [Schaefer et al. 2003] and by [Monsieur and Van Snick, 2006] comprised 104 patients.

5.3. Adverse events

No health hazards or side effects are known in conjunction with the proper administration of designated therapeutic dosages. A reversible inhibition of intestinal enzyme activity (alkaline phosphatase, sucrose and dipeptidyl peptidase) was demonstrated in animal models [Tebib, 1994; PDR for Herbal Medicines, Thomson, 2004].

Brito et al. [2008]; Mur et al. [2006]: Vine pollen allergies have been reported with *Vitis vinifera* pollen and an extract thereof In Castilla-La Mancha, Spain, the area with the highest density of vineyards in the world. Two cases of allegic reactions to *Vitis vinifera* pollen and grape have been previously reported. The aim of a prospective study by Brito et al. [2008] was to determine the clinical relevance and biochemical characteristics of vine pollen in the Spanish province of Ciudad Real. The authors designed a prospective study of patients treated in the allergy units from Puertollano and Ciudad Real for respiratory symptoms of 4 months' duration in the year 2000. Skin prick tests with a standard aeroallergen battery and *V. vinifera* pollen extract were performed on all patients. The authors also performed conjunctival and bronchial provocation tests and serum specific IgE and sodium dodecyl sulfate-polyacrylamide gel electrophoresis immunoblotting on the patients who tested positive for *Vitis vinifera* pollen.

The results of this prospective study show that *V. vinifera* pollen has a moderate clinical relevance from an allergic point of view, particularly affecting those subjects who are exposed to it during the working hours and those who perform leisure activities close to vine fields. During a period of four months, the authors performed skin prick tests to vine pollen in patients who attended the outpatient clinic with suspected respiratory allergy, finding sensitisation to *Vitis vinifera* pollen in 9 of the 200 patients seen (98 patients had allergy to other pollen). In conclusion, in areas with a high density of vineyards, vine pollen can reach midrange air concentrations and could be the cause of hay fever in those individuals with the highest level of exposure [Brito et al. 2008; Mur et al. 2006].

Assessment: In summary, there is no new safety specific information provided. No change of the safety profile is suggested.

Reports on immunoglobulin E (IgE)-mediated allergic reactions to grapes and wine are limited in the literature. Nevertheless, grapes are widely grown and consumed in Mediterranean countries. The

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object of a prospective study by Kalogeromitros et al. [2005] was to present clinical features, *in vivo* and *in vitro* allergy testing, and human leukocyte antigen (HLA) serotyping in patients with recurring reactions to grapes and grape products. Eleven unrelated Greek patients, six men and five women (aged 16-44 years; mean, 26.9 years) were enrolled based on a documented history of IgE-mediated reactions to grapes, wine, or other grape products. Their evaluation included full history, reaction severity, clinical examination, skin-prick tests with food allergens and molds, serum IgE, specific IgEs to the same allergen battery, and HLA typing. Patients reported 35 grape-induced episodes of anaphylaxis ranging from moderate to severe. The authors described several cases of repeated IgE-mediated reactions to grapes, vine and other grape products. Similar clinical manifestations in different courses of grape allergen, almost identical prevalent co-sensitisations to other already known allergenic fruits, and the first determination of HLA antigens in grape-allergic patients are the core of this study.
The authors suggest that allergy to grapes may not be as uncommon as generally believed in the literature and should be considered as a possible offending cause in certain episodes of food-induced anaphylaxis probably in genetically at-risk individuals. However, allergic reactions to *Vitis vinifera* are known and reported in rare cases [Kalogeromitros et al. 2005].

Assessment: If hypersensitivity reactions are labelled correctly in the PIL and SPC, there is no change of the safety profile of medicinal products.

[Crowell et al. 2004]: Resveratrol-Associated Renal Toxicity. Resveratrol, (3, 5, 4'-trihydoxystilbene) a compound found in grapes, mulberries, and peanuts, has antimycotic, antiviral, and beneficial cardiovascular and cancer preventive activities. To evaluate the potential toxicity of resveratrol, rats were administered by gavage 0, 300, 1000, and 3000 mg trans-resveratrol per kilogram body weight per day for 4 weeks. Most of the adverse events occurred in the rats administered 3000 mg per kilogram body weight per day. These included increased clinical signs of toxicity; reduced final body weights and food consumption; elevated BUN (blood urea nitrogen), creatinine, alkaline phosphatase, alanine aminotransferase, total bilirubin, and albumin; reduced hemoglobin, hematocrit, and red cell counts; and increased white cell counts. Increases in kidney weights and clinically significant renal lesions, including an increased incidence and severity of nephropathy, were observed. Diffuse epithelial hyperplasia in the bladder was considered, equivocal and of limited biological significance. No histological effects on the liver were observed, despite the clinical chemistry changes and increased liver weights in the females. Effects seen in the group administered 1000 mg resveratrol per kilogram body weight per day included reduced body weight gain (females only) and elevated white blood cell count (males only). Plasma resveratrol concentrations in blood collected 1 h after dose administration during week 4 were dose related but were relatively low given the high dosage levels; conjugates were not measured. Under the conditions of this study, the no observed adverse effect level was 300 mg resveratrol per kilogram body weight per day in rats (corresponding to 21 g in a 70 kg human) [Crowell et al. 2004].

Assessment: These results cannot be transferred to medical products containing Vitis vinifera or extracts thereof.

In summary, there is no safety specific information that is relevant for medical products. The reported adverse events / side effects were mild to moderate. The majority of the above mentioned events are considered to be related to underlying diseases, incidental concomitant disorders or other coincidences but not causally related to *Vitis vinifera*. From these published adverse events, no change of the safety profile of *Vitis vinifera* can be concluded. According to current knowledge, gastrointestinal disorders and hypersensitivity reactions should be labelled in the PIL and SPC.

The Periodic Safety Update Report for *Vitis vinifera* has been compiled within the joint BAH PSUR project. It summarizes safety data from 01/Nov/2003 to 01/Sep/2008 [BAH 2008].

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In the report period no new safety relevant issues were found in the literature. There were no reports, clinical or experimental studies identified that give reason for a change in the assessment of the safety and benefit/risk ratio of *Vitis vinifera*.

5.4. Serious adverse events and deaths

The case of a 51 years old female patient is reported. She was hospitalized due to an acute icterus, which had developed over 15 days and was associated with asthenia, nausea and anorexia. Beyond the mucocutaneous icterus, the clinical examination did not show any abnormality, especially no chronic hepatopathy. The laboratory parameters suggested a cytolysis due to an ALAT value that was twelve times higher than normal, γ-GT and alcalic phosphatase plasma concentrations two times higher than normal and total bilirubine of 87 µmol/L. Normal findings in the abdominal ultrasonic examination lead to the diagnosis of an acute hepatitis. The patient has a history of euthyroidism. The patient did not consume alcohol and was in a good nutritional condition. The etiologic examination of this hepatitis was negative for infections, immunological and metabolic causes. A hepatic punction-biopsy, revealed a discrete cholangitis with focal ductal hyperplasia, a major cholestasis with numerous biliary thromboses and intrahepatocytic biliary pigments, a focal hepatocellular necrosis, a lymphohistiocytic infiltrate of weak intensity without significant fibrosis. Altogether this suggested an acute cholestatic hepatitis with either viral or toxic origin. The review of the medical questionnaire revealed sporadic intake of 1 g of acetaminophen and two repeated intakes in an interval of one week, when the first symptoms had appeared. Additionally the patient took *Vitis vinifera tinctoria* (extract of red wine leaves) and Fumitory (extract of *Fumaria officinalis*) daily for two months, as "summer cure" for the treatment of heavy legs and as a stimulant. She stopped the intake of these two products three weeks prior to hospitalization, when the first general symptoms appeared. The patient had already taken *Vitis vinifera tinctoria* at the same time one year before. Fumitory was used for the first time. According to the official method used at the pharmacovigilance centres in France, this case is classified as plausible with a chronological C2 and a semiotic S3 score of two products concomitantly used with paracetamol [Bonnet et al. 2007].

Assessor's comment

This case is classified as serious because of a significant medical reaction including hospitalisation. A causal relationship with *Vitis vinifera* cannot be excluded (plausible temporal relationship). However, the used herbal products are insufficiently described; additionally relevant information on the dosage are lacking. Moreover, the concomitant use of acetaminophen must be considered. Acetaminophen is well known for its hepatotoxicity.

5.5. Laboratory findings

None reported.

5.6. Safety in special populations and situations

The product is not suitable for patients with known hypersensitivity against the herbal substance, the plant family, the herbal preparation or to the excipients of the final product.

5.7. Intrinsic (including elderly and children) /extrinsic factors

Grapevine leaf is not intended for use in children, while no restrictions are known for its use in elderly.

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5.8. Drug interactions

No drug interactions have been reported.

5.9. Use in pregnancy and lactation

Grapevine leaf should not be used during pregnancy and lactation as there are not data available.

5.10. Overdose

No information.

5.11. Drug abuse

No information.

5.12. Withdrawal and rebound

No information.

5.13. Effects on ability to drive or operate machinery or impairment of mental ability

No information.

5.14. Overall conclusions on clinical safety

The safety profile of grapevine leaf extracts can be judged as good from clinical studies and from its long term use and market availability. The available literature, on pharmacological and toxicological studies, does not give rise to safety concerns. A special focus was set on the polyphenolic constituents of the extract (proanthocyanidins, flavonoids) that can be linked with the pharmacological effects. The proof of an acceptable safety is not only given by the period of time over which this extract has been used and by the quantitative aspects of their use but also by PSURs data and the safety data obtained during the clinical trials.
Overall, *Vitis vinifera,* leaves can be considered as safe in herbal medicinal products with a positive benefit/risk ratio.

6. Overall conclusions

Well-established use

The clinical efficacy and safety of the dry aqueous extract of grapevine leaves (4-6:1) has been demonstrated in appropriately designed clinical trials and is supported by extensive use of the product in the market since 1999.

The above referred extract showed an influence to the microcirculation and transcutaneous oxygen pressure at the predominantly affected perimalleolar area of the leg in Chronic venous insufficiency (CVI) patients who were treated for six weeks. Moreover, the assayed grapevine leaves extract has shown to lead to a reduction of the volume of oedema and to improvement of the typical subjective symptoms of CVI such as tired, heavy and swollen legs or pain and tension in the legs.

Three double-blind, placebo controlled trials investigated the change in leg volume as measured by water plethysmography as the primary endpoint, another also the changes in ankle and calf

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circumference in the secondary endpoints investigated. In several of the clinical trials objective and subjective symptoms of CVI appeared to be effectively reduced, while there were some others giving negative results such as the ones of Schaefer & Petrini 2004 and Vix et al. 2006. The trials were performed according to ICH-GCP.

An additional placebo-controlled and open safety and tolerability trial provided supportive evidence for the efficacy.

This extract can therefore be regarded as an active substance with a well-established medicinal use.

The use of herbal medicinal products prepared with this extract is not recommended during pregnancy and lactation and should not be taken in children and adolescents under 18 years of age, due to the lack of adequate data.

The proposed indication is:

- Herbal medicinal product for treatment of chronic venous insufficiency, which is characterised by swollen legs, varicose veins, a feeling of heaviness, pain, tiredness, itching, tension and cramps in the calves.

Traditional use

Other extracts and herbal preparations from grapevine leaves have been widely used. The safe use of the extract can be stated on the basis of the well-known, long-lasting and traditional use of preparations of grapevine leaves in the folk medicine and as registered medicaments.. Based on the evaluation and assessment of the present documentation of the clinical efficacy and safety, the herbal substance for use as herbal tea or in other oral dosage forms, and the aqueous extracts of grapevine leaf are acceptable as traditional herbal medicinal products.

Sufficient data are available to develop a Community monograph on the well established and traditional use of *Vitis vinifera* L., leaves. The indications are suitable for self-medication. The proposed indications are:

- Traditional herbal medicinal product to relieve symptoms of discomfort and heaviness of legs related to minor venous circulatory disturbances.
- Traditional herbal medicinal product for symptomatic relief of itching and burning associated with haemorrhoids.
- Traditional herbal medicinal product for symptomatic treatment of cutaneous capillary fragility.

The use of the traditional herbal medicinal products is not recommended during pregnancy and lactation and should not be taken in children and adolescents under 18 years of age.

The minimum required data on mutagenicity (Ames test) are available for herbal preparations of grapevine leaves. **The inclusion in the Community list of traditional herbal substances and preparations is recommended.**

Annex

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EP 05 71 2607.0
Alexion Pharmaceuticals, Inc.
Our Ref.: M2367 OPP(EP)

AUXILIARY REQUEST

1. Eculizumab for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein eculizumab is to be administered by intravenous infusion to the patient under the following dosing schedule:
   (i) 600 mg each week for four weeks;
   (ii) 900 mg 1 week later;
   (iii) 900 mg on a biweekly basis; and thereafter
   (iv) 900 mg every 12 days,
   wherein said patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii), and
   wherein cell lysis is reduced to below 20% of normal serum complement activity when treated under a dosing schedule according to items (i) to (iv).

2. Eculizumab for use according to claim 1, wherein eculizumab is to be administered in combination with one or more compounds that increase hematopoiesis.

3. Eculizumab for use according to claim 2, wherein said one or more compounds that increase hematopoiesis are selected from the group consisting of: steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin, antithymocyte globulin, and antilymphocyte globulin.

4. Eculizumab for use according to any one of claims 1 to 3, wherein the patient is characterized by one or more characteristics selected from the group consisting of:
   (a) the proportion of type III red blood cells of the patient's total red blood cell count is greater than 10%;
   (b) the proportion of type III red blood cells of the patient's total red blood

cell content is greater than 25%;

(c) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 50%;

(d) the patient's platelet count is greater than 40,000 per microliter;

(e) the patient's platelet count is greater than 75,000 per microliter;

(f) the patient's platelet count is greater than 150,000 per microliter;

(g) the patient's reticulocyte count is greater than 80 x 10^9 per liter;

(h) the patient's reticulocyte count is greater than 120 x 10^9 per liter;

(i) the patient's reticulocyte count is greater than 150 x 10^9 per liter;

(j) the patient has a greater than 40% type III granulocyte clone; and

(k) the patient has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

5. Use of eculizumab in the preparation of a medicament for treating a patient with paroxysmal nocturnal hemoglobinuria (PNH), wherein eculizumab is to be administered by intravenous infusion to the patient under the following dosing schedule:

(i) 600 mg each week for four weeks;

(ii) 900 mg 1 week later;

(iii) 900 mg on a biweekly basis; and thereafter

(iv) 900 mg every 12 days,

wherein said patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii), and

wherein cell lysis is reduced to below 20% of normal serum complement activity when treated under a dosing schedule according to items (i) to (iv).

6. The use according to claim 5, wherein eculizumab is to be administered in combination with one or more compounds that increase hematopoiesis.

7 The use according to claim 6, wherein said one or more compounds that increase hematopoiesis are selected from the group consisting of: steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin,

antithymocyte globulin, and antilymphocyte globulin.

8. The use according to any one of claims 5 to 7, wherein the patient is characterized by one or more characteristics selected from the group consisting of:

(l) the proportion of type III red blood cells of the patient's total red blood cell count is greater than 10%;

(m) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 25%;

(n) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 50%;

(o) the patient's platelet count is greater than 40,000 per microliter;

(p) the patient's platelet count is greater than 75,000 per microliter;

(q) the patient's platelet count is greater than 150,000 per microliter;

(r) the patient's reticulocyte count is greater than 80 x 10^9 per liter;

(s) the patient's reticulocyte count is greater than 120 x 10^9 per liter;

(t) the patient's reticulocyte count is greater than 150 x 10^9 per liter;

(u) the patient has a greater than 40% type III granulocyte clone; and

(v) the patient has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

MAIN REQUEST

1. Eculizumab for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein eculizumab is to be administered by intravenous infusion to the patient under the following dosing schedule:
   (i) 600 mg each week for four weeks;
   (ii) 900 mg 1 week later;
   (iii) 900 mg on a biweekly basis; and thereafter
   (iv) 900 mg every 12 days,
   wherein said patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii).

2. Eculizumab for use according to claim 1, wherein eculizumab is to be administered in combination with one or more compounds that increase hematopoiesis.

3. Eculizumab for use according to claim 2, wherein said one or more compounds that increase hematopoiesis are selected from the group consisting of: steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin, antithymocyte globulin, and antilymphocyte globulin.

4. Eculizumab for use according to any one of claims 1 to 3, wherein the patient is characterized by one or more characteristics selected from the group consisting of:
   (a) the proportion of type III red blood cells of the patient's total red blood cell count is greater than 10%;
   (b) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 25%;

(c) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 50%;

(d) the patient's platelet count is greater than 40,000 per microliter;

(e) the patient's platelet count is greater than 75,000 per microliter;

(f) the patient's platelet count is greater than 150,000 per microliter;

(g) the patient's reticulocyte count is greater than 80 x 10^9 per liter;

(h) the patient's reticulocyte count is greater than 120 x 10^9 per liter;

(i) the patient's reticulocyte count is greater than 150 x 10^9 per liter;

(j) the patient has a greater than 40% type III granulocyte clone; and

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5. Use of eculizumab in the preparation of a medicament for treating a patient with paroxysmal nocturnal hemoglobinuria (PNH), wherein eculizumab is to be administered by intravenous infusion to the patient under the following dosing schedule:

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wherein said patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii).

6. The use according to claim 5, wherein eculizumab is to be administered in combination with one or more compounds that increase hematopoiesis.

7 The use according to claim 6, wherein said one or more compounds that increase hematopoiesis are selected from the group consisting of: steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin, antithymocyte globulin, and antilymphocyte globulin.

8. The use according to any one of claims 5 to 7, wherein the patient is characterized by one or more characteristics selected from the group consisting of:

(l) the proportion of type III red blood cells of the patient's total red blood cell count is greater than 10%;

(m) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 25%;

(n) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 50%;

(o) the patient's platelet count is greater than 40,000 per microliter;

(p) the patient's platelet count is greater than 75,000 per microliter;

(q) the patient's platelet count is greater than 150,000 per microliter;

(r) the patient's reticulocyte count is greater than 80 x 10^9 per liter;

(s) the patient's reticulocyte count is greater than 120 x 10^9 per liter;

(t) the patient's reticulocyte count is greater than 150 x 10^9 per liter;

(u) the patient has a greater than 40% type III granulocyte clone; and

(v) the patient has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

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Appeal number: T1897/14-3.3.04

Order

1. In accordance with the business distribution scheme of the Technical Boards of Appeal, the following shall hear the above appeal:

Chairman: G. Alt

technically qualified member: R. Morawetz

legally qualified member: K. Garnett

In the case of an extended Board:

technically qualified member: _____

legally qualified member: _____

2. The rapporteur shall be: R. Morawetz

3. The additional rapporteur shall be: _____

4. Back to the Registry for further action.

Munich, 12.11.14

The Chairwoman G. Alt

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Europäisches Patentamt
Herr Eric Bihl
Abteilung CDR

80295 München

Epoline - Mailbox und MyFiles:
Umstellung aller Akten auf Zusammenschluss Nr. 31

München, 07. Januar 2015
BE/TWI

Sehr geehrter Herr Bihl,

wir möchten Sie hiermit beauftragen, eine durchgängige Umstellung aller Akten auf unseren Zusammenschluss Nr. 31 durchzuführen. Außerdem bitten wir Sie, zukünftige Anmeldungen diesbezüglich zu überprüfen und auf unseren Zusammenschluss umzustellen, falls dies nicht der Fall ist.

Unsere Adresse soll bitte zukünftig einheitlich wie folgt in Ihrer Datenbank angegeben werden:

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Siebertstr. 3
81675 München

Wir bitten Sie um eine Bestätigung nach erfolgter Aktenumstellung per Email an twi@vossiusandpartner.com.

Mit freundlichen Grüßen

Dr. Natalia Berryman

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VOSSIUS & PARTNER Fax:+498941304111 7 Jan 2015 15:09 P001/001

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Patentanwälte Rechtsanwälte mbB

EPO - Munich
58
12. März 2015

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European Patent Office

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Mitteilung neue Gesellschaftsform
VOSSIUS & PARTNER
Patentanwälte Rechtsanwälte mbB
Unser Zeichen: GF

München, 12. März 2015
BE/SKA

Sehr geehrte Damen und Herren,

hiermit teilen wir mit, dass sich die Gesellschaftsform unserer Kanzlei geändert hat in eine Partnerschaft mit beschränkter Berufshaftung. Der neue Name der Kanzlei lautet nun:

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Es wird gebeten, eine entsprechende Umschreibung der Vertreterdaten (Zusammenschluss 31) durchzuführen.

Dr. Natalia Berryman
European Patent Attorney

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80298 MUNICH
GERMANY
Tel. +49 (0)89 2399-0
Fax +49 (0)89 2399-4465

Appeal number: T1897/14-3.3.04

Order

Change of the composition of the Board

compare to order of 12.11.14

1.

Chairman: G. Alt

technically qualified member: R. Morawetz

technically qualified member: _____

legally qualified member: L. Bühler

legally qualified member: _____

2. The rapporteur shall be: R. Morawetz

3. The additional rapporteur shall be: _____

4. Reasons:
Change in the business distribution scheme of the board for the current year.

5. Back to the Registry for further action.

Munich, 20.07.15

The Chairwoman G. Alt

EPO Form 3303.16

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Appeal number: **T1897/14-3.3.04**

Order

Oral proceedings are to be held

on 03.05.18

at 09:30 hours

Room N° 0124

Munich 24.07.17

The Chairwoman
G. Alt

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ALLEMAGNE

Date
27.07.17

Zeichen/Reference/Référence	Anmeldung Nr./Application No./Demande n°//Patent Nr./Patent No./Brevet n°
M2367 EP APPR	05712607.0 / 1720571
Anmelder/Applicant/Demandeur//Patentinhaber/Proprietor/Titulaire	
ALEXION PHARMACEUTICALS, INC.	

Appeal number: **T1897/14-3.3.04**

Summons to oral proceedings pursuant to Rule 115(1) EPC

You are hereby summoned to the oral proceedings concerning the above appeal.
The proceedings are scheduled to take place

on 03.05.18 at 09:30 hrs in Room 0124

Bob-van-Benthem-Platz 1, 80469 Munich (DE)

The proceedings will be public.

Room 115 is available as a waiting-room.

You are requested to attend outside the appointed room **10 minutes** before the hearing.

Registered letter with advice of delivery

Samsung Bioepis Exhibit No. 1027
Page 486

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Appeal number: **T1897/14-3.3.04**

You are reminded that

- if a party who has been duly summoned to oral proceedings does not appear, the proceedings may continue without that party (Rule 115(2) EPC).
- oral proceedings will only be postponed at the request of a party for serious reasons (see Notice in Special edition No. 3 OJ EPO 2007, 115).
- as regards filing authorisations for representatives or company employees, see Decision of the President of the EPO dated 12.7.2007 on the filing of authorisations in Special edition No. 3 OJ EPO 2007, 128.
- concerning the language of the proceedings, attention is drawn to Rule 4 EPC. Notice given pursuant to Rule 4(1) EPC before the first instance is not valid in proceedings before the Boards of Appeal.
- According to Rule 103(2) EPC it is possible to obtain a 50% reimbursement of the appeal fee if the appeal is withdrawn under certain circumstances (OJ EPO 2014, A3).

Composition of the Board

Chairman: G. Alt

Member: R. Morawetz
Member: L. Bühler

For all urgent communications in connection with the oral proceedings please use <u>only</u> EPO Online Services (preferably) or fax No. + 49 (0) 89 2399 3014.

If you are planning not to attend the oral proceedings or if you are aware of any matter that could have a bearing on the appointment of interpreters, you are requested to inform the Board of this, preferably in writing, at the earliest possible opportunity.

The Registrar P. Cremona
Tel.: 089 / 2399 - 3341

Annex(es): ☑ Confirmation of receipt Form 3936
☐ Communication pursuant to Article 15(1) of the Rules of Procedure of the Boards of Appeal
☐

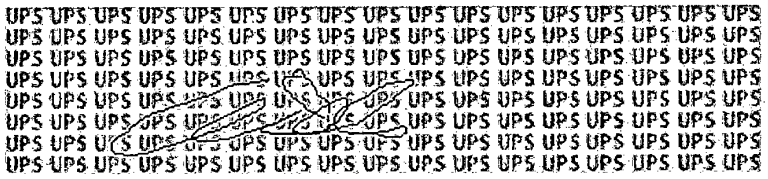
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Page 487

Tracking Information

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Name:	Vossius & Partner
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Country:	DE
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Send Date:	Thursday, July 27, 2017

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Boards of Appeal

The Registry EPO - Munich
Name: P. Cremona 100
Tel.: 089 / 2399 - 341 31. Juli 2017

Date: 27.07.17

Zeichen/Reference/Référence	Anmeldung Nr./Application No./Demande n°//Patent Nr./Patent No./Brevet n°
M2367 OPP(EP) APPR	05712607.0 / 1720571
Anmelder/Applicant/Demandeur//Patentinhaber/Proprietor/Titulaire	
ALEXION PHARMACEUTICALS, INC.	

Appeal number: T1897/14-3.3.04

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Empfangsbescheinigung über den Zugang des vorstehend bezeichneten Schriftstücks
Acknowledgement of receipt of the document specified above
Récépissé du document spécifié ci-dessus

Unter Bezugnahme auf die Mitteilung im ABl. EPA 7/2010, 377 wird gebeten, die Empfangsbescheinigung mit Empfangsdatum und Unterschrift zu versehen und **umgehend** an das EPA zurückzusenden:

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Appeal number: T1897/14-3.3.04

Order

Oral proceedings to be held on 03.05.18

☐ are cancelled.

☑ are rescheduled for 03.05.18 at 09:30 hours

Room N° OP02

Munich, 13.09.17

The Chairwoman G. Alt

Samsung Bioepis Exhibit No. 1027
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Datum / Date
18.09.2017

Zeichen/Reference/Référence		Anmeldung Nr. / Application No. / Demande n°. // Patent Nr. / Patent No. / Brevet n°.
M2367 EP	APPR	05712607.0 // 1720571
Anmelder / Applicant / Demandeur // Patentinhaber / Proprietor / Titulaire		
ALEXION PHARMACEUTICALS, INC.		

Appeal number: **T1897/14-3.3.04**

Communication of the Registry

The Registrar P. Cremona
Tel.: 089 / 2399 - 3341

Registered letter with advice of delivery

This document: 2 page(s) including this page

Annex(es): EPO Form 3936

Communication text

Move of the Boards of Appeal to Haar/ Important information for oral proceedings

The oral proceedings appointed for 3 May 2018 will now take place in:

Room OP02
Richard-Reitzner-Allee 8
85540 HAAR

Samsung Bioepis Exhibit No. 1027
Page 491

A main waiting area is located at the entrance, directly after the security check. Further waiting rooms are located near to the oral proceedings rooms.

The other details (date, time etc.) in the original summons remain unchanged.

Reason: The Boards of Appeal will by then have moved to a new building in Haar. Oral proceedings will therefore be conducted there.

Please also consult the EPO website for information on travel to the Haar building, parking facilities, hotel accommodation, location map and on-site security arrangements.

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EPO - Munich
101
20. Sep. 2017

Boards of Appeal

The Registry
Name: P. Cremona
Tel.: 089 / 2399 - 3341

Date: 18.09.17

Zeichen/Reference/Référence	Anmeldung Nr./Application No./Demande n°//Patent Nr./Patent No./Brevet n°
M2367 EP APPR	05712607.0 / 1720571
Anmelder/Applicant/Demandeur//Patentinhaber/Proprietor/Titulaire	
ALEXION PHARMACEUTICALS, INC.	

Appeal number: T1897/14-3.3.04

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19. SEP. 2017

Unterschrift / Signature:

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T1897/14-3304

05712607.0

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09. Nov. 2017

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European Patent Office

80298 M U N I C H

T1897/14-3.3.04
(05 71 2607.0 / 1720571)
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Vossius Ref.: M2367 OPP(EP)

Appeal Number T1551/12-3.3.01
EP 07 75 3249.7
Alexion Pharmaceuticals, Inc.
Vossius Ref.: P2913 EP

EP 16 17 2335.8 – 1466
Alexion Pharmaceuticals, Inc.
Vossius Ref.: P2913 EP/2

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

EP 16 20 1244.7 - 1410
Alexion Pharmaceuticals, Inc.
Vossius Ref.: R1249 EP/3

München, November 9, 2017
WA/FLG/MUA

We herewith resign from representation of the five above-identified matters.

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European Patent Attorneys
European Trademark Attorneys
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Josef Schmidt, Dipl.-Ing. 1) 9)
Dr. Hans-Rainer Jaenichen, Dipl.-Biol. 1)
Dr. Alexa v. Uexküll, M.Sc. 1)
Dr. Rudolf Weinberger, Dipl.-Chem. 1)
Dr. Joachim Wachenfeld, Biol. 1)
Dr. Friederike Stolzenburg, Dipl.-Biol. 1)
Rainer Viktor, Dipl.-Ing. 1)
Dr. Natalia Berryman, Dipl.-Chem. 1)
Dr. Jürgen Meier, Dipl.-Biol. 1)
Dr. Stefan Fickert, Dipl.-Chem., LL.M. 1)
Arnold Asmussen, Dipl.-Ing. (FH) 1)
Elard Schenck zu Schweinsberg, Dipl.-Ing., LL.M. 1)
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Switzerland: HR Firmennummer CH-270.9.001.271-9

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It is respectfully requested that this change is registered in the official Register.

Dr. Joachim Wachenfeld
European Patent Attorney

Samsung Bioepis Exhibit No. 1027
Page 497

Europäisches Patentamt
European Patent Office
Office européen des brevets

European Patent Office
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GERMANY

Questions about this communication ?
Contact Customer Services at www.epo.org/contact

J A Kemp
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ROYAUME UNI

Date
20.11.17

Reference	Application No./Patent No.
	05712607.0 - 1412 / 1720571
Applicant/Proprietor	
ALEXION PHARMACEUTICALS, INC.	

Communication of amended entries concerning the representative (R. 143(1)(h) EPC)

As requested, for the above-mentioned European patent application / European patent the entries concerning the representative have been amended as follows:

J A Kemp
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The amendment will be recorded in the Register of European Patents.

For the Opposition Division

Samsung Bioepis Exhibit No. 1027
Page 498

J A • K E M P

BY ONLINE FILING

The European Patent Office
Bayerstrasse 34
(entrance via Zollstrasse 3)
80335 Munich
Germany

29 March 2018

Dear Sirs

Appeal No. T1897/14-3.3.04
Opposition to European Patent No. 1720571
(Application No. 05712607.0)
ALEXION PHARMACEUTICALS, INC.
Our Ref: C412290EP PXT

I hereby withdraw the Appeal filed by Alexion Pharmaceuticals Inc.

Yours faithfully

Electronically Signed
DR PAMELA M TUXWORTH
ASSOCIATION NO. 811

PATENT ATTORNEYS • TRADE MARK ATTORNEYS
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A list of our partners is available at our principal place of business at the address above. Regulated by IPREG

Europäisches Patentamt
European Patent Office
Office européen des brevets

Submission in opposition proceedings

Representative:

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- representing the proprietor(s):	Alexion Pharmaceuticals, Inc.
Proprietor/representative's reference	C412290EP

The information given below is pertaining to the following patent in opposition proceedings:

Patent No.	EP1720571
Application No.	EP05712607.0
Date of mention of the grant in the European Patent Bulletin (Art. 97(3), Art. 99(1) EPC)	13 June 2012
Title of the invention	METHOD OF TREATING HEMOLYTIC DISEASE
Proprietor of the patent	Alexion Pharmaceuticals, Inc.

Documents attached:

	Description of document	Original file name	Assigned file name
1	Any annexes (other than citation) to an opposition letter - Request to withdraw appeal	Withdraw the Appeal C412290EP PXT.PDF	OTHER-1.PDF

Signatures

Place:	
Date:	**29 March 2018**
Signed by:	**Elizabeth Davenport 55598**
Association:	**J A Kemp**
Representative name:	**DAVENPORT, Elizabeth**
Capacity:	**(Representative)**

Samsung Bioepis Exhibit No. 1027
Page 501

Europäisches Patentamt
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Acknowledgement of receipt

We hereby acknowledge receipt of the following submission by the proprietor:

Submission number	6191091
Application number	EP05712607.0
Patent number	EP1720571
Date of receipt	29 March 2018
Your reference	C412290EP
Proprietor	Alexion Pharmaceuticals, Inc.
Title	METHOD OF TREATING HEMOLYTIC DISEASE
Documents submitted	package-data.xml ep-oppo.pdf (2 p.) ep-opposition-data.xml OTHER-1.PDF\Withdraw the Appeal C412290EP PXT.PDF (1 p.)
Submitted by	CN=Elizabeth Davenport 55598
Method of submission	Online
Date and time receipt generated	29 March 2018, 16:49 (CEST)
Message Digest	5D:0B:34:04:C2:8E:7F:73:6F:82:BB:60:1A:18:5E:B6:61:51:2C:B6

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

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Appeal number: T1897/14-3.3.04

Order

Oral proceedings to be held on 03.05.18

☑ are cancelled.

☐ are rescheduled for at hours

Room N°

Munich, 03.04.18

The Chairwoman G. Alt

Samsung Bioepis Exhibit No. 1027
Page 503

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Datum / Date
05.04.2018

Zeichen/Reference/Référence	Anmeldung Nr. / Application No. / Demande n°. // Patent Nr. / Patent No. / Brevet n°.
APPR	05712607.0 // 1720571
Anmelder / Applicant / Demandeur // Patentinhaber / Proprietor / Titulaire	
ALEXION PHARMACEUTICALS, INC.	

Appeal number: **T1897/14-3.3.04**

Communication of the Board of Appeal

The Rapporteur R. Morawetz
The Registrar P. Cremona
Tel.: 089 / 2399 - 3341

Registered letter

This document: 7 page(s) including this page

Annex(es):

Communication text

FAX: 089 413 04 111 (29 March 2018)

1. This communication is sent pursuant to Article 15(1) of the Rules of Procedure of the Boards of Appeal (RPBA). Any opinions expressed are provisional and not binding on the board in arriving at its decision (Article 17(2) RPBA).

2. The purpose of this communication is to set out some of the issues to be considered during the oral proceedings.

3. An opposition was filed against the patent. The patent was opposed under Article 100(a) EPC on the grounds of lack of novelty (Article 54 EPC) and lack of inventive step (Article 56 EPC) and under Article 100(b) and 100 (c) EPC. The opponent withdrew its opposition with letter dated 7 October 2013 and thus ceased to be a party to the proceedings.

4. The opposition division continued the opposition proceedings of its own volition. It decided that neither the main request nor the auxiliary request before it met the requirements of Article 56 EPC and revoked the patent.

5. The appeal of the patent proprietor (hereinafter "appellant") lies against this decision.

6. The board adopts the numbering of the documents as set out in point 1 of the decision under appeal (documents D1 to D15). Documents D16 to D19 were submitted by the appellant with its statement of grounds of appeal.

7. The appellant requests that the decision under appeal be set aside and that a patent be granted on the basis of the main request or, alternatively, on the basis of the auxiliary request, both as submitted with the statement of grounds of appeal. These claim requests correspond to the claim requests underlying the decision under appeal.

8. At present the board has the following comments on the claim requests on file.

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Page 505

Main request

Article 56 EPC - claim 1

9. The subject-matter of claim 1 relates to a four-stage dosing schedule for treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH) with the anti-C5 antibody eculizumab.

10. The opposition division held that the subject-matter of claim 1 of the main request lacked an inventive step over the teaching of document D5 as closest prior art when combined with the disclosure of document D6 (see decision under appeal, points 2.10 to 2.27).

11. The appellant did not dispute that document D5 was the closest prior art. According to the appellant the differences between the teaching of document D5 and the claimed subject-matter was twofold: (i) document D5 was silent about the dosage schedule applied prior to the bi-weekly administration of 900 mg and (ii) document D5 was silent about the feature of administering 900 mg every 12 days (see statement of grounds of appeal, point 1.3).

According to the appellant the problem to be solved was the provision of a complete eculizumab dosage regimen which is capable of preventing complement breakthroughs in all PNH patients (ibid.).

12. The appellant submitted that the opposition division had applied an "ex post facto" analysis in selecting document D6 as secondary reference (see statement of grounds of appeal, point 1.4.2), that optimising the eculizumab dosing schedule was not a matter of routine (ibid., point 1.5.1), and that document D5 failed to render decreasing the dosing interval from 14 days to 12 days obvious (ibid., point 1.5.3).

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13. At present, the board is not convinced by the arguments submitted by the appellant.

14. Document D5 reports the results obtained in an extension trial, after the patients had completed a pilot trial. Thus, document D5 discloses that "*we previously reported that eculizumab was very effective at acutely controlling intravascular hemolysis in a 3-month Pilot study of transfusion-dependent PNH patients. We now report that all 11 patients from the initial study entered an extension trial of 900 mg eculizumab by i.v. infusion over 30 minutes every other week for one year. To date all patients remain on eculizumab, completing between 11 and 15 months of therapy*" (see abstract, page 509, lines 5 to 8).

15. In the board's view, the skilled person faced with the problem of having to provide a complete eculizumab dosage regimen and knowing from document D5 that the patients receiving 900 mg every other week during the extension study were on a pilot study before would be motivated to look for the dosage regimen used in the pilot study.

16. Documents D2, D3 and D6 all relate to initial studies carried out in PNH patients which patients are said to take part in an extension study. Thus, document D2 reports that "*patients received 600 mg of eculizumab each week for four weeks, after which the dose was switched to 900 mg every two weeks. Ten patients have completed the 12-week treatment, and all choose to enter a 12-month extension trial*" (see second and first paragraph from bottom). Document D3 reports that "*eleven transfusion-dependent PNH patients have completed a 3-month pilot study receiving 600 mg eculizumab by intravenous infusion over 30 min weekly for four doses and then 900 mg every other week (...) all patients have continued on eculizumab in an*

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extension study" (see page 87, abstract, lines 5 to 8 and lines 28 to 30). Document D6 discloses "*Eleven transfusion-dependent PNH patients have completed an open-label pilot study of eculizumab. Patients received 600 mg eculizumab by intravenous infusion over 30 min weekly for four doses, followed by 900 mg 1 week later, and then 900 mg every other week for the remainder of the trial. All 11 patients completed the study and are currently participating in an open-label extension study*" (see page 197, abstract, lines 8 to 15).

17. Documents D2 and D3 thus disclose the same dosage regimen (600 mg eculizumab weekly for four doses and then 900 mg every other week) while the dosage regimen disclosed in document D6 contains an extra step compared to the dosage regimen disclosed in documents D2 and D3 (600 mg eculizumab weekly for four doses, followed by 900 mg 1 week later, and then 900 mg every other week) and is thus more detailed.

18. In the board's opinion, the skilled person faced with the problem of having to provide a complete dosing regimen and faced with the very similar disclosures in documents D2, D3 and D6 which apparently relate to one and the same pilot study would choose the most comprehensive dosage regimen, i.e. the one disclosed in document D6. No ex post facto considerations are involved in this choice.

19. The appellant relied on documents D13, D15, D7, D16, D17, D14 to support its argument that the skilled person would have proceeded very cautiously when either increasing the dose or reducing the dose of eculizumab (see statement of grounds of appeal, point 1.5.1).

20. The board notes that documents D14, D15, D16, D17 were not available before the priority date of the patent and could thus not have influenced the skilled person's

Samsung Bioepis Exhibit No. 1027
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attitude.

21. Appellant's arguments based on documents D7 and D13 fail because document D6 discloses that the dosage regimen is well tolerated "*with no patients reporting a serious adverse event*" (see page 197, abstract, lines 15 to 18).

22. As regards the second difference (see point 11), the board notes that document D5 already discloses that "*two patients experienced transient breakthroughs in complement activity associated with increased LDH, hemoglobinuria and mild symptoms; these resolved immediately upon next dose of eculizumab and further breakthroughs were prevented by a slight decrease in the dosing interval*" (see abstract, page 510a, lines 8 to 12).

23. Document D5 thus discloses that a slight decrease of the dosing interval solves the problem of complement breakthrough. All that the skilled person thus needed to do was to maintain the dose (900 mg) and decrease the dosing interval from "every other week" to slightly less than that until complement breakthrough was prevented. Since the skilled person already knew from document D5 that this would give the desired result, the actual determination of the appropriate dosing interval, i.e. every 12 days, would appear to be a matter of routine devoid of inventive activity.

Auxiliary request

Article 56 EPC - claim 1

24. The opposition division held that the feature "*wherein cell lysis is reduced below 20% of normal serum complement activity when treated under a dosing schedule according to items (i) to (iv)*" was at best

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another read-out to confirm the same resolution of breakthrough already recorded in document D5.

25. The appellant submitted that the effective inhibition of haemolytic activity was a key advantage being associated with the subject-matter claimed in the main and auxiliary requests and that this advantage was expressly incorporated in the main claim of the auxiliary request (ibid., point II).

26. At present, the board fails to see how this line of reasoning addresses the reasons given in the decision under appeal, it appears rather to confirm the point made by the opposition division. In any case, since the subject-matter claimed in the main request is at present considered to be obvious, the board fails to see how the inclusion of an effect associated with a dosage regimen which is considered to be obvious can render the claimed subject-matter inventive.

Concluding remarks

27. Should the appellant choose to file further written submissions, then attention is drawn to Article 114(2) EPC and Articles 13(1) and 13(3) RPBA.

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Date
06.04.18

Zeichen/Reference/Référence	Anmeldung Nr./Application No./Demande n°//Patent Nr./Patent No./Brevet n°
APPR	05712607.0 / 1720571
Anmelder/Applicant/Demandeur//Patentinhaber/Proprietor/Titulaire	
ALEXION PHARMACEUTICALS, INC.	

Appeal number: T1897/14-3.3.04

Cancellation

1. ☑ The oral proceedings appointed for 03.05.18 have been cancelled.

2. ☐ The oral proceedings appointed for

have been rescheduled to: at .

will now be in Room No.

The other provisions in the summons of 27.07.17 remain unchanged.

The Registrar P. Cremona
Tel.: 089 / 2399 - 3341

Annex(es): ☑ Acknowledgement of receipt - EPO Form 3936

Registered letter with advice of delivery

Europäisches Patentamt
European Patent Office
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Termination of opposition proceedings of patent no. 05712607.0 - 1116/ 1720571 with revocation of the patent 13.04.18

I. **Findings**

1. The opposition proceedings are terminated for the following reason:
   The patent was revoked

   REVO 01.07.14

   The time limit pursuant to Article 108 EPC has expired.
   No appeal or application pursuant to Article 122 EPC has been filed.

   REVO 2 and DEAD coded and Form 2362 despatched.

2. Fees situation:

OPPO04	010	00625522	12.03.13	EUR	745,00
NOAP04	011	00116739	08.09.14	EUR	1 860,00
CLMS(2)	015	00571629	01.09.06	EUR	765,00

   Checked for correct fee payment. Refund(s) made on ...../... .

3. Any models still to hand were returned on ....n/a..... .

4. Enter "DEAD" and dispose of paper file

13.04.2018 ..... Date

Bertoni, Sara ..... Formalities officer

SB51929

EPO Form 2358.2 02.15

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Boards of Appeal

The Registry

Name: P. Cremona

Tel.: 089 / 2399 - 3341

Date: 06.04.18

Zeichen/Reference/Référence	Anmeldung Nr./Application No./Demande n°//Patent Nr./Patent No./Brevet n°
APPR	05712607.0 / 1720571
Anmelder/Applicant/Demandeur//Patentinhaber/Proprietor/Titulaire	
ALEXION PHARMACEUTICALS, INC.	

Appeal number: T1897/14-3.3.04

EPA/EPO/OEB Formblatt/Form/Formulaire: 3017

Empfangsbescheinigung über den Zugang des vorstehend bezeichneten Schriftstücks
Acknowledgement of receipt of the document specified above
Récépissé du document spécifié ci-dessus

Unter Bezugnahme auf die Mitteilung im ABl. EPA 7/2010, 377 wird gebeten, die Empfangsbescheinigung mit Empfangsdatum und Unterschrift zu versehen und **umgehend** an das EPA zurückzusenden:

With reference to the Notice in OJ EPO 7/2010, 377, you are requested to date and sign the acknowledgement of receipt and return it to the EPO **immediately:**

Conformément au communiqué paru au JO OEB 7/2010, 377, vous êtes prié d'indiquer sur le récépissé la date de réception du document, de signer le récépissé et de le renvoyer **sans délai** à l' OEB:

- **über die Online-Dienste des EPA** (als Anlage zu EPA Form 1038) / **through EPO Online Services** (as annex to EPO Form 1038) / **par les services en ligne de l'OEB** (en tant que pièce jointe au formulaire OEB 1038),
- **per Fax / by fax / par téléfax (+49 (0) 89 2399-4465 or +31 (0)70 340-3016)**
- oder per Post / or by post / ou par courrier

Empfangen am / Received on / Reçu le:

13. 4. 18

Unterschrift / Signature:

Empfangsberechtigter/authorised recipient/
le destinataire ou la personne dûment mandatée

Rücksende-Adresse / Return address / Adresse de retour
(Umschlag / envelope / enveloppe ISO C4 / DL / C6/C5 / C6)

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Received at the EPO on Apr 13, 2018 10:48:30. Page 1 of 1
uration: 04.13.2018 10:47:05 AM - 04.13.2018 10:48:30 AM. This page 1 of 1 was completed at 04.13.2018 10:48:30 AM

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Datum/Date
18.04.18

Zeichen/Reference/Référence	Anmeldung Nr./Application No./Demande n°//Patent Nr./Patent No./Brevet n°
C412290EP APPR	05712607.0 / 1720571
Anmelder/Applicant/Demandeur//Patentinhaber/Proprietor/Titulaire	
ALEXION PHARMACEUTICALS, INC.	

Appeal number: T1897/14-3.3.04

Closure of the appeal proceedings
Withdrawal of the appeal/application/opposition by the sole appellant

The

☑ appeal(s) ☐ opposition(s)

☐ application

of the appellant has been withdrawn by letter dated 29.03.18 and received on 29.03.18 .

The Board has instructed the Registrar to notify all concerned that the appeal proceedings are accordingly closed without a substantive decision.

The appeal fee will be reimbursed pursuant to Rule 103(2)(a) EPC.

By order of the Board of Appeal
The Registrar Danielle Hampe
Tel.: 089 / 2399 - 3341

Annex(es):/

Registered letter

EPO Form 3324

Europäisches Patentamt
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Date
20.04.18

Zeichen/Reference/Référence	Anmeldung Nr./Application No./Demande n°//Patent Nr./Patent No./Brevet n°
C412290EP APPR	05712607.0 / 1720571
Anmelder/Applicant/Demandeur//Patentinhaber/Proprietor/Titulaire	
ALEXION PHARMACEUTICALS, INC.	

Appeal number: T1897/14-3.3.04

Refund of fees

The following fees was/were paid in respect of the application 05712607.0 :

Fee	Reference	Voucher No	Date	Currency	Amount
appeal-fee	011	00116739	08.09.14	EUR	1.860,00

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

CREDITING THE DEPOSIT ACCOUNT 28050038 (J.A. Kemp)

Amount refundable:	Reference	Currency	Amount
930,00	011	EUR	930,00

Reason: pursuant to R.103(2)(a) EPC, following the withdrawal of the appeal filed on 28.03.2018.

The Registrar D. Hampe
Tel.: 089 / 2399 - 3341

Annex(es): ☐

Beschwerdekammern des Europäischen Patentamts ★ Boards of Appeal of the European Patent Office ★ Chambres de recours de l'Office européen des brevets