HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use SOLIRIS safely and effectively. See full prescribing information for SOLIRIS.

Soliris® (eculizumab) injection, for intravenous use

Initial U.S. Approval: 2007

WARNING: SERIOUS MENINGOCOCCAL INFECTIONS

See full prescribing information for complete boxed warning

Life-threatening and fatal meningococcal infections have occurred in patients treated with Soliris and may become rapidly life-threatening or fatal if not recognized and treated early (5.1).

- **Comply with the most current Advisory Committee on Immunization Practices (ACIP) recommendations for meningococcal vaccination in patients with complement deficiencies (5.1).**
- **Immunize patients with meningococcal vaccines at least 2 weeks prior to administering the first dose of Soliris, unless the risks of delaying Soliris therapy outweigh the risk of developing a meningococcal infection. [see *Warnings and Precautions (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).

INDICATIONS AND USAGE

Soliris is a complement inhibitor indicated for:

- The treatment of patients with paroxysmal nocturnal hemoglobinuria (PNH) to reduce hemolysis (1.1).
- The treatment of patients with atypical hemolytic uremic syndrome (aHUS) to inhibit complement-mediated thrombotic microangiopathy (1.2).

Limitation of Use

Soliris is not indicated for the treatment of patients with Shiga toxin *E. coli* related hemolytic uremic syndrome (STEC-HUS).

DOSAGE AND ADMINISTRATION

Only administer as an intravenous infusion

PNH Dosage Regimen: (2.1)
aHUS Dosage Regimen: (2.2)

D24

DOSAGE FORMS AND STRENGTHS

300 mg single-dose 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 (≥20%) are: headache, diarrhea, hypertension, upper respiratory infection, abdominal pain, vomiting, nasopharyngitis, anemia, cough, peripheral edema, nausea, urinary tract infections, pyrexia (6.1).

To report SUSPECTED ADVERSE REACTIONS, contact Alexion Pharmaceuticals, Inc. at) 1-844-259-6783 or FDA at 1-800-FDA-1088 or www.fda.gov/medwatch.

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).

See 17 PATIENT COUNSELING INFORMATION and Medication Guide.

Revised: 01/2017

FULL PRESCRIBING INFORMATION: CONTENTS*

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FULL PRESCRIBING INFORMATION

WARNING: SERIOUS MENINGOCOCCAL INFECTIONS

Life-threatening and fatal meningococcal infections have occurred in patients treated with Soliris. Meningococcal infection may become rapidly life-threatening or fatal if not recognized and treated early [*see Warnings and Precautions* (5.1)].

- **Comply with the most current Advisory Committee on Immunization Practices (ACIP) recommendations for meningococcal vaccination in patients with complement deficiencies.**
- **Immunize patients with meningococcal vaccines least 2 weeks prior to administering the first dose of Soliris, unless the risks of delaying Soliris therapy outweigh the risk of developing a meningococcal infection. [see *Warnings and Precautions (5.1)* for additional guidance on the management of the risk of meningococcal infection].**
- **Monitor patients for early signs of meningococcal infections and evaluate immediately if infection is suspected.**

Soliris is available only through a restricted program under a Risk Evaluation and Mitigation Strategy (REMS). Under the Soliris REMS, prescribers must enroll in the program [*see Warnings and Precautions (5.2)*]. Enrollment in the Soliris REMS program and additional information are available by telephone: 1-888-SOLIRIS (1-888-765-4747) or at solirisrems.com.

1 INDICATIONS AND USAGE

1.1 Paroxysmal Nocturnal Hemoglobinuria (PNH)

Soliris is indicated for the treatment of patients with paroxysmal nocturnal hemoglobinuria (PNH) to reduce hemolysis.

1.2 Atypical Hemolytic Uremic Syndrome (aHUS)

Soliris is indicated for the treatment of patients with atypical hemolytic uremic syndrome (aHUS) to inhibit complement-mediated thrombotic microangiopathy.

Limitation of Use

Soliris is not indicated for the treatment of patients with Shiga toxin *E. coli* related hemolytic uremic syndrome (STEC-HUS).

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.

Administer Soliris 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

Administer Soliris at the recommended dosage regimen time points, or within two days of these time points.

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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 infusion of fresh frozen plasma	60 minutes prior to each infusion of fresh frozen plasma

2.3 Preparation and Administration

Dilute Soliris to a final admixture concentration of 5 mg/mL using the following steps:

- Withdraw the required amount of Soliris from the vial into a sterile syringe.
- Transfer the recommended dose to an infusion bag.
- Dilute Soliris to a final concentration of 5 mg/mL by adding the appropriate amount (equal volume of diluent to drug volume) of 0.9% Sodium Chloride Injection, USP; 0.45% Sodium Chloride Injection, USP; 5% Dextrose in Water Injection, USP; or Ringer's Injection, USP to the infusion bag.

The final admixed Soliris 5 mg/mL infusion volume is 60 mL for 300 mg doses, 120 mL for 600 mg doses, 180 mL for 900 mg doses or 240 mL for 1200 mg doses (Table 3).

Table 3: Preparation and Reconstitution of Soliris

Soliris Dose	Diluent Volume	Final Volume
300 mg	30 mL	60 mL
600 mg	60 mL	120 mL
900 mg	90 mL	180 mL
1200 mg	120 mL	240 mL

Gently invert the infusion bag containing the diluted Soliris solution to ensure thorough mixing of the product and diluent. Discard any unused portion left in a vial, as the product contains no preservatives.

Prior to administration, the admixture should be allowed to adjust to room temperature [18°-25° C, 64-77° F]. The admixture must not be heated in a microwave or with any heat source other than ambient air temperature.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

2.4 Administration

Do Not Administer As An Intravenous Push or Bolus Injection

Administer the Soliris admixture by intravenous infusion over 35 minutes in adults and 1 to 4 hours in pediatric patients via gravity feed, a syringe-type pump, or an infusion 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 in adults. Monitor the patient for at least one hour following completion of the infusion for signs or symptoms of an infusion reaction.

3 DOSAGE FORMS AND STRENGTHS

Soliris is supplied as 300 mg single-dose 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

Life-threatening and fatal meningococcal infections have occurred in patients treated with Soliris. The use of Soliris increases a patient's susceptibility to serious meningococcal infections (septicemia and/or meningitis).

Vaccinate for meningococcal disease according to the most current Advisory Committee on Immunization Practices (ACIP) recommendations for patients with complement deficiencies. Revaccinate patients in accordance with ACIP recommendations, considering the duration of Soliris therapy.

Immunize patients without a history of meningococcal vaccination at least 2 weeks prior to receiving the first dose of Soliris. If urgent Soliris therapy is indicated in an unvaccinated patient, administer meningococcal vaccine(s) as soon as possible.

In prospective clinical studies, 75/100 patients with aHUS were treated with Soliris less than 2 weeks after meningococcal vaccination and 64 of these 75 patients received antibiotics for prophylaxis of meningococcal infection until at least 2 weeks after meningococcal vaccination. The benefits and risks of antibiotic prophylaxis for prevention of meningococcal infections in patients receiving Soliris have not been established.

Vaccination reduces, but does not eliminate, the risk of meningococcal infections. In clinical studies, 2 out of 196 PNH patients developed serious meningococcal infections while receiving treatment with Soliris; both had been vaccinated [*see Adverse Reactions (6.1)*]. In clinical studies among non-PNH patients, meningococcal meningitis occurred in one unvaccinated patient. In addition, 3 out of 130 previously vaccinated patients with aHUS developed meningococcal infections while receiving treatment with Soliris [*see Adverse Reactions (6.1)*].

Closely monitor patients for early signs and symptoms of meningococcal infection and evaluate patients immediately if an infection is suspected. Meningococcal infection may become rapidly life-threatening or fatal if not recognized and treated early. Discontinue Soliris in patients who are undergoing treatment for serious meningococcal infections.

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) or at solirisrems.com.

5.3 Other Infections

Soliris blocks terminal complement activation; therefore patients may have increased susceptibility to infections, especially with encapsulated bacteria. Additionally, Aspergillus infections have occurred in immunocompromised and neutropenic patients. Children treated with Soliris may be at increased risk of developing serious infections due to *Streptococcus pneumoniae* and *Haemophilus 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 [*see Warnings and Precautions (5.1)*].

5.4 Monitoring Disease Manifestations 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 trials, 18 patients (5 in the prospective studies) discontinued Soliris treatment. TMA complications occurred following a missed dose in 5 patients, and Soliris was reinitiated in 4 of these 5 patients.

Clinical signs and symptoms of TMA include changes in mental status, seizures, angina, dyspnea, or thrombosis. In addition, the following changes in laboratory parameters may identify a TMA complication: occurrence of two, or repeated measurement of any one of the following: a decrease in platelet count by 25% or more compared to baseline or the peak platelet count during Soliris treatment; an increase in serum creatinine by 25% or more compared to baseline or nadir during Soliris treatment; or, an increase in serum LDH by 25% or more over baseline or nadir during Soliris treatment.

If TMA complications occur after Soliris discontinuation, consider reinstitution of Soliris treatment, plasma therapy [plasmapheresis, plasma exchange, or fresh frozen plasma infusion (PE/PI)], or appropriate organ-specific supportive measures.

5.5 Thrombosis Prevention and Management

The effect of withdrawal of anticoagulant therapy during Soliris treatment has not been established. Therefore, treatment with Soliris should not alter anticoagulant management.

5.6 Infusion Reactions

As with all protein products, administration of Soliris may result in infusion reactions, including anaphylaxis or other hypersensitivity reactions. In clinical trials, no patients experienced an infusion reaction which required discontinuation of Soliris. Interrupt Soliris infusion and institute appropriate supportive measures if signs of cardiovascular instability or respiratory compromise occur.

6 ADVERSE REACTIONS

The following serious adverse reactions are discussed in greater detail in other sections of the labeling:

- Serious Meningococcal Infections [*see Warnings and Precautions (5.1)*]
- Other Infections [*see Warnings and Precautions (5.3)*]
- Monitoring Disease Manifestations After Soliris Discontinuation [*see Warnings and Precautions (5.4)*]
- Thrombosis Prevention and Management [*see Warnings and Precautions (5.5)*]
- Infusion Reactions [*see Warnings and Precautions (5.6)*]

6.1 Clinical Trial Experience

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice.

Meningococcal infections are the most important adverse reactions experienced by patients receiving Soliris. In PNH clinical studies, two patients experienced meningococcal sepsis. Both patients had previously received a meningococcal vaccine. In clinical studies among patients without PNH, meningococcal meningitis occurred in one unvaccinated patient. Meningococcal sepsis occurred in one previously vaccinated patient enrolled in the retrospective aHUS study during the post-study follow-up period [*see Warnings and Precautions (5.1)*].

PNH

The data described below reflect exposure to Soliris in 196 adult patients with PNH, age 18-85, of whom 55% were female. All had signs or symptoms of intravascular hemolysis. Soliris was studied in a placebo-controlled clinical study (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.

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 four prospective, single-arm studies, three in adult and adolescent patients (aHUS Studies 1, 2, and 4), one in pediatric and adolescent patients (aHUS Study 5) and one retrospective study (aHUS Study 3).

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The data described below were derived from 78 adult and adolescent patients with aHUS enrolled in aHUS Study 1, aHUS Study 2, and aHUS Study 4. All patients received the recommended dosage of Soliris. Median exposure was 67 weeks (range: 2-145 weeks). Table 5 summarizes all adverse events reported in at least 10% of patients in aHUS Studies 1, 2, and 4 combined.

Table 5: Per Patient Incidence of Adverse Events in 10% or More Adult and Adolescent Patients Enrolled in aHUS Study 1, aHUS Study 2 and aHUS Study 4 Separately and in Total

MedDRA ver. 15.1	Number (%) of Patients			
	Study 1 (n=17)	Study 2 (n=20)	Study 4 (n=41)	Total (n=78)
Vascular Disorders				
Hypertension[a]	10 (59)	9 (45)	7 (17)	26 (33)
Hypotension	2 (12)	4 (20)	7 (17)	13 (17)
Infections and Infestations				
Bronchitis	3 (18)	2 (10)	4 (10)	9 (12)
Nasopharyngitis	3 (18)	11 (55)	7 (17)	21 (27)
Gastroenteritis	3 (18)	4 (20)	2 (5)	9 (12)
Upper respiratory tract infection	5 (29)	8 (40)	2 (5)	15 (19)
Urinary tract infection	6 (35.3)	3 (15)	8 (20)	17 (22)
Gastrointestinal Disorders				
Diarrhea	8 (47)	8 (40)	12 (32)	29 (37)
Vomiting	8 (47)	9 (45)	6 (15)	23 (30)
Nausea	5 (29)	8 (40)	5 (12)	18 (23)
Abdominal pain	3 (18)	6 (30)	6 (15)	15 (19)
Nervous System Disorders				
Headache	7 (41)	10 (50)	15 (37)	32 (41)
Blood and Lymphatic System Disorders				
Anemia	6 (35)	7 (35)	7 (17)	20 (26)
Leukopenia	4 (24)	3 (15)	5 (12)	12 (15)
Psychiatric Disorders				
Insomnia	4 (24)	2 (10)	5 (12)	11 (14)
Renal and Urinary Disorders				
Renal impairment	5 (29)	3 (15)	6 (15)	14 (18)
Proteinuria	2 (12)	1 (5)	5 (12)	8 (10)
Respiratory, Thoracic and Mediastinal Disorders				
Cough	4 (24)	6 (30)	8 (20)	18 (23)
General Disorders and Administration Site Conditions				
Fatigue	3 (18)	4 (20)	3 (7)	10 (13)
Peripheral edema	5 (29)	4 (20)	9 (22)	18 (23)
Pyrexia	4 (24)	5 (25)	7 (17)	16 (21)
Asthenia	3 (18)	4 (20)	6 (15)	13 (17)
Eye Disorder	5 (29)	2 (10)	8 (20)	15 (19)
Metabolism and Nutrition Disorders				
Hypokalaemia	3 (18)	2 (10)	4 (10)	9 (12)
Neoplasms benign, malignant, and unspecified (including cysts and polyps)	1 (6)	6 (30)	1 (20)	8 (10)
Skin and Subcutaneous Tissue Disorders				
Rash	2 (12)	3 (15)	6 (15)	11 (14)
Pruritis	1 (6)	3 (15)	4 (10)	8 (10)
Musculoskeletal and Connective Tissue Disorders				
Arthralgia	1 (6)	2 (10)	7 (17)	10 (13)
Back pain	3 (18)	3 (15)	2 (5)	8 (10)

[a] includes the preferred terms hypertension, accelerated hypertension, and malignant hypertension.

In aHUS Studies 1, 2, and 4 combined, 60% (47/78) of patients experienced a serious adverse event (SAE). The most commonly reported SAEs were infections (24%), hypertension (5%), chronic renal failure (5%), and renal impairment (5%). Five patients discontinued Soliris due to adverse events; three due to worsening renal function, one due to new diagnosis of Systemic Lupus Erythematosus, and one due to meningoccal meningitis.

aHUS Study 5 included 22 pediatric and adolescent patients, of which 18 patients were less than 12 years of age. All patients received the recommended dosage of Soliris. Median exposure was 44 weeks (range: 1 dose-87 weeks). Table 6 summarizes all adverse events reported in at least 10% of patients enrolled in aHUS Study 5.

Table 6: Per Patient Incidence of Adverse Reactions in 10% or More Patients Enrolled in aHUS Study 5

MedDRA ver. 15.1	1 month to <12 yrs (n=18)	Total (n=22)
Eye Disorders	3 (17)	3 (14)
Gastrointestinal Disorders		
Abdominal pain	6 (33)	7 (32)
Diarrhoea	5 (28)	7 (32)
Vomiting	4 (22)	6 (27)
Dyspepsia	0	3 (14)
General Disorders and Administration Site Conditions		
Pyrexia	9 (50)	11 (50)
Infections and Infestations		
Upper respiratory tract infection	5 (28)	7 (32)
Nasopharyngitis	3 (17)	6 (27)
Rhinitis	4 (22)	4 (18)
Urinary Tract infection	3 (17)	4 (18)
Catheter site infection	3 (17)	3 (14)
Musculoskeletal and Connective Tissue Disorders		
Muscle spasms	2 (11)	3 (14)
Nervous System Disorders		
Headache	3 (17)	4 (18)
Renal and Urinary Disorders	3 (17)	4 (18)
Respiratory, Thoracic and Mediastinal Disorders		
Cough	7 (39)	8 (36)
Oropharyngeal pain	1 (6)	3 (14)
Skin and Subcutaneous Tissue Disorders		
Rash	4 (22)	4 (18)
Vascular Disorders		
Hypertension	4 (22)	4 (18)

In aHUS Study 5, 59% (13/22) of patients experienced a serious adverse event (SAE). The most commonly reported SAEs were hypertension (9%), viral gastroenteritis (9%), pyrexia (9%), and upper respiratory infection (9%). One patient discontinued Soliris due to an adverse event (severe agitation).

Analysis of retrospectively collected adverse event data from pediatric and adult patients enrolled in 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 7.

Table 7: 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 (40)	1 (25)	9 (47)
Gastrointestinal Disorders				
Diarrhea	1 (20)	4 (40)	1 (25)	6 (32)
Vomiting	2 (40)	1 (10)	1 (25)	4 (21)
Infections and Infestations				
Upper respiratory tract infection[a]	2 (40)	3 (30)	1 (25)	6 (32)
Respiratory, Thoracic and Mediastinal Disorders				
Cough	3 (60)	2 (20)	0 (0)	5 (26)
Nasal congestion	2 (40)	2 (20)	0 (0)	4 (21)
Cardiac Disorders				
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 with eculizumab. The immunogenicity of Soliris has been evaluated using two different immunoassays for the detection of anti-eculizumab antibodies: a direct enzyme-linked immunosorbent assay (ELISA) using the Fab fragment of eculizumab as target was used for the PNH indication; and an electro-chemiluminescence (ECL) bridging assay using the eculizumab whole molecule as target was used for the aHUS indication, as well as for additional patients with PNH. In the PNH population, antibodies to Soliris were detected in 3/196 (2%) patients with PNH treated with Soliris using the ELISA assay and in 5/161 (3%) patients treated with Soliris using the ECL assay. In patients with aHUS treated with Soliris, antibodies to Soliris were detected in 3/100 (3%) using 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 3 patients with aHUS and also for the 5 patients with PNH with positive samples using the ECL assay. 2/161 patients in the PNH group (1.2%) and 1/100 patients in the aHUS group (1%) had low positive values for neutralizing antibodies. No apparent correlation of antibody development to clinical response was observed in either indication. 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 and 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

The following adverse reactions have been identified during post-approval use of Soliris. Because these reactions are reported voluntarily from a population of uncertain size, it is not always possible to reliably estimate their frequency or establish a causal relationship to Soliris exposure.

Cases of serious or fatal meningococcal infections have been reported.

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Pregnancy Category C

Risk Summary

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 Data

Animal reproduction studies were conducted in mice using doses of a murine anti-C5 antibody that approximated 2-4 times (low dose) and 4-8 times (high dose) the recommended human Soliris dose, based on a body weight comparison. When animal exposure to the antibody occurred in the time period from before mating until early gestation, no decrease in fertility or reproductive performance was observed. When maternal exposure to the antibody occurred during organogenesis, two cases of retinal dysplasia and one case of umbilical hernia were observed among 230 offspring born to mothers exposed to the higher antibody dose; however, the exposure did not increase fetal loss or neonatal death. When maternal exposure to the antibody occurred in the time period from implantation through weaning, a higher number of male offspring became moribund or died (1/25 controls, 2/25 low dose group, 5/25 high dose group). Surviving offspring had normal development and reproductive function.

8.3 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

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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 have not been established.

The safety and effectiveness of Soliris for the treatment of aHUS have been established in pediatric patients. Use of Soliris in pediatric patients for this indication is supported by evidence from four adequate and well-controlled clinical studies assessing the safety and effectiveness of Soliris for the treatment of aHUS. The studies included a total of 47 pediatric patients (ages 2 months to 17 years). The safety and effectiveness of Soliris for the treatment of aHUS appear similar in pediatric and adult patients [*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

Nineteen patients 65 years of age or older (15 with PNH and 4 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-dose 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 Dosage and Administration (2.2)*].

Dedicated studies have not been conducted to evaluate the PK of Soliris in special patient populations identified by gender, race, age (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 Paroxysmal Nocturnal Hemoglobinuria (PNH)

The safety and efficacy of Soliris in PNH patients with hemolysis were assessed in a randomized, double-blind, placebo-controlled 26 week study (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 8).

Table 8: 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 9). 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 9: PNH Study 1 Results

	Placebo N = 44	Soliris N = 43
Percentage of patients with stabilized hemoglobin levels	0	49
Packed RBC units transfused per patient (median)	10	0
(range)	(2 - 21)	(0 - 16)
Transfusion avoidance (%)	0	51
LDH levels at end of study (median, U/L)	2,167	239
Free hemoglobin at end of study (median, mg/dL)	62	5

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 Atypical Hemolytic Uremic Syndrome (aHUS)

Five single-arm studies [four prospective (aHUS Studies 1, 2, 4 and 5) 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 and Study 5 was based on body weight [*see Dosage and Administration (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 were required to have ADAMTS13 activity level above 5%; observed range of values in the trial were 70%-121%. Seventy-six percent of patients had an identified complement regulatory factor mutation or auto-antibody. Table 10 summarizes the key baseline clinical and disease-related characteristics of patients enrolled in aHUS Study 1.

Table 10: 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. In aHUS Study 1, the median duration of Soliris therapy was approximately 100 weeks (range: 2 weeks to 145 weeks).

Renal function, as measured by eGFR, was improved and maintained during Soliris therapy. The mean eGFR (± SD) increased from 23 ± 15 mL/min/1.73m² at baseline to 56 ± 40 mL/min/1.73m² by 26 weeks; this effect was maintained through 2 years (56 ± 30 mL/min/1.73m²). Four of the five patients who required dialysis at baseline were able to discontinue dialysis.

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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 (± SD) increased from 109 ± 32 x10^9/L at baseline to 169 ± 72 x10^9/L by one week; this effect was maintained through 26 weeks (210 ± 68 x10^9/L), and 2 years (205 ± 46 x10^9/L). When treatment was continued for more than 26 weeks, two additional patients achieved Hematologic Normalization as well as Complete TMA response. Hematologic Normalization and Complete TMA response were maintained by all responders. In aHUS Study 1, responses to Soliris were similar in patients with and without identified mutations in genes encoding complement regulatory factor proteins.

Table 11 summarizes the efficacy results for aHUS Study 1.

Table 11: Efficacy Results for aHUS Study 1

Efficacy Parameter	aHUS Study 1 at 26 wks[1] N = 17	aHUS Study 1 at 2 yrs[2] N = 17
Complete TMA response, n (%)	11 (65)	13 (77)
Median Duration of complete TMA response, weeks (range)	38 (25, 56)	99 (25, 139)
eGFR improvement ≥15 mL/min/1.73 m^2, n (%)	9 (53)	10 (59)
Median duration of eGFR improvement, days (range)	251 (70, 392)	ND
Hematologic normalization, n (%)	13 (76)	15 (88)
Median Duration of hematologic normalization, weeks (range)	37 (25, 62)	99 (25, 145)
TMA event-free status, n (%)	15 (88)	15 (88)
Daily TMA intervention rate, median (range)		
Before eculizumab	0.82 (0.04, 1.52)	0.82 (0.04, 1.52)
On eculizumab treatment	0 (0, 0.31)	0 (0, 0.36)

[1] At data cut-off (September 8, 2010).

[2] At data cut-off (April 20, 2012).

aHUS Sensitive to PE/PI (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 were required to have ADAMTS13 activity level above 5%; observed range of values in the trial were 37%-118%. Seventy percent of patients had an identified complement regulatory factor mutation or auto-antibody. Table 12 summarizes the key baseline clinical and disease-related characteristics of patients enrolled in aHUS Study 2.

Table 12: 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. In aHUS Study 2, the median duration of Soliris therapy was approximately 114 weeks (range: 26 to 129 weeks).

Renal function, as measured by eGFR, was maintained during Soliris therapy. The mean eGFR (± SD) was 31 ± 19 mL/min/1.73m^2 at baseline, and was maintained through 26 weeks (37 ± 21 mL/min/1.73m^2) and 2 years (40 ± 18 mL/min/1.73m^2). No patient required new dialysis with Soliris.

Reduction in terminal complement activity was observed in all patients after the commencement of Soliris. Soliris reduced signs of complement-mediated TMA activity, as shown by an increase in mean platelet counts from baseline to 26 weeks. Platelet counts were maintained at normal levels despite the elimination of PE/PI. The mean platelet count (± SD) was 228 ± 78 x 10^9/L at baseline, 233 ± 69 x 10^9/L at week 26, and 224 ± 52 x 10^9/L at 2 years. When treatment was continued for more than 26 weeks, six additional patients achieved Complete TMA response. Complete TMA Response and Hematologic Normalization were maintained by all responders. In aHUS Study 2, responses to Soliris were similar in patients with and without identified mutations in genes encoding complement regulatory factor proteins.

Table 13 summarizes the efficacy results for aHUS Study 2.

Table 13: Efficacy Results for aHUS Study 2

Efficacy Parameter	aHUS Study 2 at 26 wks[1] N = 20	aHUS Study 2 at 2 yrs[2] N = 20
Complete TMA response, n (%)	5 (25)	11 (55)
Median duration of complete TMA response, weeks (range)	32 (12, 38)	68 (38, 109)
eGFR improvement ≥15 mL/min/1.73 m^2, n (%)	1 (5)	8 (40)
TMA Event-free status n (%)	16 (80)	19 (95)
Daily TMA intervention rate, median (range)		
Before eculizumab	0.23 (0.05, 1.07)	0.23 (0.05, 1.07)
On eculizumab treatment	0	0 (0, 0.01)
Hematologic normalization[4], n (%)	18 (90)	18 (90)
Median duration of hematologic normalization, weeks (range)[3]	38 (22, 52)	114 (33, 125)

[1] At data cut-off (September 8, 2010).

[2] At data cut-off (April 20, 2012).

[3] Calculated at each post-dose day of measurement (excluding Days 1 to 4) using a repeated measurement ANOVA model.

[4] In 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.

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 (± SD) increased from 171 ± 83 x10^9/L at baseline to 233 ±109 x10^9/L after one week of therapy; this effect was maintained through 26 weeks (mean platelet count (± SD) at week 26: 254 ± 79 x10^9/L).

A total of 19 pediatric patients (ages 2 months to 17 years) received Soliris in 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 <18 years of age (n=4). Fifty three percent of pediatric patients had an identified complement regulatory factor mutation or auto-antibody. Overall, the efficacy results for these pediatric patients appeared consistent with what was observed in patients enrolled in aHUS Studies 1 and 2 (Table 14). No pediatric patient required new dialysis during treatment with Soliris.

Table 14: 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)
Complete TMA response, n (%)	2 (40)	5 (50)	1 (25)	8 (42)
Patients with eGFR improvement ≥ 15 mL/min/1.73 m^2, n (%)[2]	2 (40)	6 (60)	1 (25)	9 (47)
Platelet count normalization, n (%)[1]	4 (80)	10 (100)	3 (75)	17 (89)
Hematologic Normalization, n (%)	2 (40)	5 (50)	1 (25)	8 (42)
Daily TMA intervention rate, median (range)				
Before eculizumab	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)

[1] Platelet count normalization was defined as a platelet count of at least 150,000 X 10^9/L on at least two consecutive measurements spanning a period of at least 4 weeks.

[2] Of the 9 patients who experienced an eGFR improvement of at least 15 mL/min/1.73 m^2, one received dialysis throughout the study period and another received Soliris as prophylaxis following renal allograft transplantation.

Adult Patients with aHUS (aHUS Study 4)

aHUS Study 4 enrolled patients who displayed signs of thrombotic microangiopathy (TMA). In order to qualify for enrollment, patients were required to have a platelet count < lower limit of normal range (LLN), evidence of hemolysis such as an elevation in serum LDH, and serum creatinine above the upper limits of normal, without the need for chronic dialysis. The median patient age was 35 (range: 18 to 80 years). All patients enrolled in aHUS Study 4 were required to have ADAMTS13 activity level above 5%; observed range of values in the trial were 28%-116%. Fifty-one percent of patients had an identified complement regulatory factor mutation or auto-antibody. A total of 35 patients received PE/PI prior to eculizumab. Table 15 summarizes the key baseline clinical and disease-related characteristics of patients enrolled in aHUS Study 4.

Table 15: Baseline Characteristics of Patients Enrolled in aHUS Study 4

Parameter	aHUS Study 4 N = 41
Time from aHUS diagnosis until start of study drug in months, median (range)	0.79 (0.03 – 311)
Time from current clinical TMA manifestation until first study dose in months, median (range)	0.52 (0.03-19)
Baseline platelet count (× 10^9/L), median (range)	125 (16 – 332)
Baseline LDH (U/L), median (range)	375 (131 – 3318)

Patients in aHUS Study 4 received Soliris for a minimum of 26 weeks. In aHUS Study 4, the median duration of Soliris therapy was approximately 50 weeks (range: 13 weeks to 86 weeks).

Renal function, as measured by eGFR, was improved during Soliris therapy. The mean eGFR (± SD) increased from 17 ± 12 mL/min/1.73m^2 at baseline to 47 ± 24 mL/min/1.73m^2 by 26 weeks. Twenty of the 24 patients who required dialysis at study baseline were able to discontinue dialysis during Soliris treatment.

Reduction in terminal complement activity and an increase in platelet count relative to baseline were observed after commencement of Soliris. Soliris reduced signs of complement-mediated TMA activity, as shown by an increase in mean platelet counts from baseline to 26 weeks. In aHUS Study 4, mean platelet count (± SD) increased from 119 ± 66 x10^9/L at baseline to 200 ± 84 x10^9/L by one week; this effect was maintained through 26 weeks (mean platelet count (± SD) at week 26: 252 ± 70 x10^9/L). In aHUS Study 4, responses to Soliris were similar in patients with and without identified mutations in genes encoding complement regulatory factor proteins or auto-antibodies to factor H.

Table 16 summarizes the efficacy results for aHUS Study 4.

Table 16: Efficacy Results for aHUS Study 4

Efficacy Parameter	aHUS Study 4 (N = 41)
Complete TMA response, n (%),	23 (56),
95% CI	40,72
Median duration of complete TMA response, weeks (range)	42 (6, 75)
Patients with eGFR improvement ≥ 15 mL/min/1.73 m2, n (%)	22 (54)
Hematologic Normalization, n (%)	36 (88)
Median duration of hematologic normalization, weeks (range)	46 (10, 75)
TMA Event-free Status, n (%)	37 (90)
Daily TMA Intervention Rate, median (range)	
Before eculizumab	0.63 (0, 1.38)
On eculizumab treatment	0 (0, 0.58)

Pediatric and Adolescent Patients with aHUS (aHUS Study 5)

aHUS Study 5 enrolled patients who were required to have a platelet count < lower limit of normal range (LLN), evidence of hemolysis such as an elevation in serum LDH above the upper limits of normal, serum creatinine level ≥97 percentile for age without the need for chronic dialysis. The median patient age was 6.5 (range: 5 months to 17 years). Patients enrolled in aHUS Study 5 were required to have ADAMTS13 activity level above 5%;observed range of values in the trial were 38%-121%. Fifty percent of patients had an identified complement regulatory factor mutation or auto-antibody. A total of 10 patients received PE/PI prior to eculizumab. Table 17 summarizes the key baseline clinical and disease-related characteristics of patients enrolled in aHUS Study 5.

Table 17: Baseline Characteristics of Patients Enrolled in aHUS Study 5

Parameter	Patients 1 month to <12 years (N = 18)	All Patients (N = 22)
Time from aHUS diagnosis until start of study drug in months, median (range)	0.51 (0.03 – 58)	0.56 (0.03-191)
Time from current clinical TMA manifestation until first study dose in months, median (range)	0.23 (0.03 – 4)	0.2 (0.03-4)
Baseline platelet count (x 10^9/L), median (range)	110 (19-146)	91 (19-146)
Baseline LDH (U/L) median (range)	1510 (282-7164)	1244 (282-7164)

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Patients in aHUS Study 5 received Soliris for a minimum of 26 weeks. In aHUS Study 5, the median duration of Soliris therapy was approximately 44 weeks (range: 1 dose to 88 weeks).

Renal function, as measured by eGFR, was improved during Soliris therapy. The mean eGFR (± SD) increased from 33 ± 30 mL/min/1.73m^2 at baseline to 98 ± 44 mL/min/1.73m^2 by 26 weeks. Among the 20 patients with a CKD stage ≥2 at baseline, 17 (85%) achieved a CKD improvement of ≥1 stage. Among the 16 patients ages 1 month to <12 years with a CKD stage ≥2 at baseline, 14 (88%) achieved a CKD improvement by ≥1 stage. Nine of the 11 patients who required dialysis at study baseline were able to discontinue dialysis during Soliris treatment. Responses were observed across all ages from 5 months to 17 years of age.

Reduction in terminal complement activity was observed in all patients after commencement of Soliris. Soliris reduced signs of complement-mediated TMA activity, as shown by an increase in mean platelet counts from baseline to 26 weeks. The mean platelet count (± SD) increased from 88 ± 42 x10^9/L at baseline to 281 ± 123 x10^9/L by one week; this effect was maintained through 26 weeks (mean platelet count (±SD) at week 26: 293 ± 106 x10^9/L). In aHUS Study 5, responses to Soliris were similar in patients with and without identified mutations in genes encoding complement regulatory factor proteins or auto-antibodies to factor H.

Table 18 summarizes the efficacy results for aHUS Study 5.

Table 18: Efficacy Results for aHUS Study 5

Efficacy Parameter	Patients 1 month to <12 years (N = 18)	All Patients (N = 22)
Complete TMA response, n (%)	11 (61)	14 (64)
95% CI	36, 83	41, 83
Median Duration of complete TMA response, weeks (range)[1]	40 (14, 77)	37 (14, 77)
eGFR improvement ≥15 mL/min/ 1.73•m^2•n (%)	16 (89)	19 (86)
Complete Hematologic Normalization, n (%)	14 (78)	18 (82)
Median Duration of complete hematologic normalization, weeks (range)	38 (14, 77)	38 (14, 77)
TMA Event-Free Status, n (%)	17 (94)	21 (95)
Daily TMA Intervention rate, median (range)		
Before eculizumab treatment	0.2 (0, 1.7)	0.4 (0, 1.7)
On eculizumab treatment	0 (0, 0.01)	0 (0, 0.01)

[1] through data cutoff (October 12, 2012).

16 HOW SUPPLIED/STORAGE AND HANDLING

Soliris (eculizumab) is supplied as 300 mg single-dose vials containing 30 mL of 10 mg/mL sterile, preservative-free Soliris solution per vial.

Store Soliris vials in the original carton until time of use under refrigerated conditions at 2-8° C (36-46° F) and protected from light. Soliris vials may be held in the original carton at controlled room temperature (not more than 25° C/77° F) for only a single period up to 3 days.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

Advise the patient to read FDA-approved patient labeling (Medication Guide).

Meningococcal Infection

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.

Inform patients that they are required to receive 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. Inform patients that vaccination may not prevent meningococcal infection *[see Warnings and Precautions (5.1)]*.

Signs and Symptoms of Meningococcal Infection

Inform patients about the signs and symptoms of meningococcal infection, and strongly advise patients 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
- fever and a rash
- confusion
- muscle aches with flu-like symptoms
- eyes sensitive to light

Inform patients 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.

Other Infections

Inform patients that there may be an increased risk of other types of infections, particularly those due to encapsulated bacteria. Additionally, Aspergillus infections have occured in immunocompromised and neutropenic patients. Inform parents or caregivers of children receiving Soliris for the treatment of aHUS that their child should be vaccinated against *Streptococcus pneumoniae* and *Haemophilus influenza* type b (Hib) according to current medical guidelines.

Discontinuation

Inform patients with PNH 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. Inform patients with aHUS 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. Inform patients who discontinue Soliris 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.
100 College Street
New Haven, CT 06510 USA

US License Number 1743

This product, or its use, may be covered by one or more US patents, including US 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 chance 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 meningococcal vaccination at least 2 weeks before your first dose of Soliris unless you have already had this vaccine. If your doctor decided that urgent treatment with Soliris is needed, you should receive meningococcal vaccination as soon as possible.**
2. **If you had a meningococcal vaccine in the past, you might need additional vaccination before starting Soliris. Your doctor will decide if you need additional meningococcal vaccination.**
3. **Meningococcal vaccines do 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**
   - **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 *Haemophilis 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 medications 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 in adults and 1-4 hours in pediatric patients. 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 cell count
  - 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:

- headache
- diarrhea
- hypertension
- common cold (upper respiratory infection)
- abdominal pain
- vomiting
- nasopharyngitis
- anemia
- cough
- peripheral edema
- nausea
- urinary tract infections
- pyrexia

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.

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BRIEF REPORT

Recurrence of Neisserial Meningococcemia Due to Deficiency of Terminal Complement Component

MAJ JOSEPH T. MORRIS and MAJ WILLIAM J. KELLY, MC, USA, Fort Sam Houston, Tex

INHERITED AND ACQUIRED DEFECTS of the complement system predispose susceptible individuals to infections with *Neisseria meningitidis*.[1,2] Deficiencies of the terminal complement components (C5, C6, C7, or C8) are mainly associated with mild recurrent meningococcal infections in patients usually more than 10 years old at the time of the first infection.[1,2] The prevalence of complement deficiencies in patients with meningococcal disease ranges from 0 to 30%,[1,3,4] whereas in a white general population the prevalence is estimated to be less than 0.03%.[1,2] *Neisseria meningitidis* infections due to the common serogroups (A, B, C) are seldom associated with complement deficiencies.[1,2,4] Rasmussen et al[5] found no complement deficiencies in any of 132 patients with serogroup B infections. We report the case of a patient with recurrent neisserial meningococcemia due to deficiency of the sixth component of complement.

CASE REPORT

A 21-year-old black man who had had no pertinent past illnesses, came to the emergency room complaining of a severe headache. During the evaluation his mental status deteriorated to the point that he responded only to noxious stimuli. He was subsequently given 4 million units of penicillin G for meningitis and was admitted. His admission temperature was 38.3°C (101°F) rectally, pulse rate 50/min, blood pressure 160/98 mm Hg, and respiratory rate 14/min. Both pupils measured 5 mm and responded sluggishly to light. Nuchal rigidity and Kernig's and Brudzinki's signs were present. Results of lung, cardiac, and abdominal examinations were normal. Petechiae measuring 1 to 2 mm covered the chest, legs, and soles of the feet. Serum chemistry values were sodium 136, potassium 4, chloride 101, and bicarbonate 20 mEq/L; glucose 152, BUN 14, and creatinine 1.4 mg/dL. WBC was 21 600/mm^3 with 67% neutrophils and 22% band forms, and the hematocrit value was 45%. Lumbar puncture showed an opening pressure of 50 cm, and Gram's stain of the purulent CSF showed multiple gram-negative diplococci consistent with *Neisseria meningitidis*. The CSF protein value was 660 and glucose value, 0 mg/dL. Penicillin G therapy was continued at 2 million units every 2 hours intravenously, and 2 doses of methylprednisolone were given (2 g every 6 hours). The patient's mental status returned to normal over the following 72 hours. He received a 14-day course of penicillin G, and rifampin was given to his roommates, household contacts, and girlfriend. The *N meningitidis* was stereotyped as group B. There was no evidence of adrenal insufficiency at discharge from the hospital.

The patient did well until 3 weeks after discharge when he was admitted for evaluation of diffuse myalgias, right-sided neck stiffness, sore throat, and a temperature of 39°C (102.3°F). Physical examination showed no nuchal rigidity or Kernig's sign. WBC was 19 300/mm^3 with a left shift. Lumbar puncture yielded CSF containing 3 WBCs and 13 RBCs/mm^3, with a glucose value of 95 and a protein value of 17 mg/dL. Over the next 24 hours increasing myalgias developed, in addition to palatal petechiae and purpura on the palms and soles. He was given penicillin, gentamicin, and methylprednisolone for possible Waterhouse-Friderichsen syndrome. Blood cultures grew *N meningitidis* group B. Complement determinations showed normal C3, C4, C5, C7, and C8 levels but a CH_{50} of 0 CH U/mL and a greatly reduced C6 level of <1.5 mg/dL (normal 4.8 to 7.2 mg/dL). The patient received a 14-day course of penicillin G for treatment of recurrent neisserial bacteremia. He also received pneumococcal and *Haemophilus influenzae* vaccinations and 2 days of rifampin therapy for elimination of a possible *N meningitidis* carrier state. Methylprednisolone therapy was discontinued after 48 hours when no signs of Waterhouse-Friderichsen syndrome appeared.

DISCUSSION

The alternate and classical pathways of the complement system are important components of the host's immune response to infections caused by encapsulated organisms. Deficiencies of complement components have been associated with recurrent infections due to specific organisms. A number of studies have demonstrated the association of complement deficiencies and *Neisseria meningitidis* infections.[4,6-12] Our patient has C6 deficiency as a cause of his recurrent bacteremia with *Neisseria meningitidis* serogroup B. He had had no previous episodes of neisserial infections.

The median age of first infection in patients with late manifestation of complement deficiency has been reported to be 17 years, despite the presence of the defect since birth and the early peak age-specific incidence of meningococcal disease in the general population (ages 3 to 8 months).[2] Recurrent infections are not unusual with discrete recurrences reported in 45% of complement deficient individuals.[2]

Our patient had recurrent infections with *Neisseria meningitidis* serogroup B. This is an infrequent situation, since recurrent infections with *Neisseria meningitidis* are more commonly caused by serogroup Y than by the common serogroup B.

SUMMARY

Recurrent infections with *Neisseria meningitidis* are attributed to deficiencies of terminal complement components. The sero-

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The views of the authors do not necessarily reflect the position of the Department of the Army or the Department of Defense.

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type most commonly responsible for recurrent *N meningitidis* infections is serotype Y. We have reported a case of recurrent meningitis due to *N meningitidis* in a patient who was found to be deficient in the sixth component of complement. Complement deficiencies should be considered in any patient with recurrent infections caused by *N meningitidis*.

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D4

Eculizumab

Prop Inn; USAN

Humanized Anti-C5 Monoclonal Antibody

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

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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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ADVANCES IN HEMATOLOGY

Current Developments in the Management of Hematologic Disorders

Section Editor: Craig M. Kessler, MD

Eculizumab for the Treatment of Paroxysmal Nocturnal Hemoglobinuria

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H&O Can you give a short background on the standard of care for paroxysmal nocturnal hemoglobinuria before the advent of eculizumab?

AH Until recently, the treatment for paroxysmal nocturnal hemoglobinuria (PNH) was predominantly supportive. Red blood cell (RBC) transfusions were administered when appropriate, and warfarin or other anticoagulation therapy was given following a thrombosis. In the UK we practice primary prophylaxis with anticoagulant therapy depending on the neutrophil PNH clone size. Other therapies, such as corticosteroids or androgen therapy, have also been used, but less commonly and with efficacy in only a few patients; the mechanism of action behind these treatments is unclear. Stem cell transplantation has also been used in the past, but of course this approach is associated with serious complications. It has been difficult to develop an evidence base for treatment because of the heterogeneous nature and rarity of this disease. Before eculizumab (Alexion), treatment for PNH was limited to alleviating symptoms and improving patient quality of life. Eculizumab is a monoclonal antibody that blocks the complement system at protein C5.

H&O What is the rationale for the development of eculizumab for the treatment of PNH?

AH We know the hemolysis in PNH is complement-mediated, thus this drug should be effective in preventing hemolysis. The pathogenesis of PNH is known to be due to complement sensitivity of RBCs against complement attack. RBCs in PNH have lost their protective proteins (eg, CD55 and CD59) that would normally prevent complement-mediated lysis. It was hypothesized that if the complement attack on the RBCs could be blocked, the hemolysis in PNH and its associated symptoms could be prevented. Based on patients who have inherited complement deficiencies, it appears that patients with deficiencies in complement proteins C5–C9 suffer few clinical consequences compared to those with deficiencies in the complement proteins before C5. Therefore, C5 seemed to be a logical target for blocking the complement cascade. Eculizumab was developed for the purpose of blocking the complement system safely and without severe complications.

H&O Eculizumab has been evaluated for the treatment of rheumatoid arthritis, systemic lupus erythematosus, and other autoimmune conditions. Is there a similarity between these conditions and PNH that spurred investigators to evaluate eculizumab in PNH?

AH The research strategy has been to develop therapeutic approaches that block complement activity, which contributes to a variety of disorders. There is some evidence that some components of autoimmune disorders such as rheumatoid arthritis and systemic lupus erythematosus are complement-mediated. In PNH, however, almost all of the clinical symptoms are due to the uncontrolled activity of complement. Therefore, whereas there are many possible disorders in which complement blockade might have some efficacy, PNH is the disorder in which we had little doubt that eculizumab would be effective.

H&O What were some of the initial findings with eculizumab in PNH?

AH In May 2002, we began the pilot study of eculizumab in PNH. Patients enrolled had been having regular transfusions, and most exhibited dark urine (a sign of intravascular hemolysis) and severe symptoms of PNH such as difficulty swallowing (dysphagia) and abdominal pain. Men complained of erectile dysfunction. Additionally, they had biochemical parameters indicative of active, ongoing intravascular hemolysis. We found that just 1 infusion of the drug caused a resolution of the

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dark urine by the next morning. Other symptoms, such as abdominal pain and difficulty in swallowing, resolved quickly. Of the 6 male patients who had experienced erectile dysfunction, 5 experienced complete resolution of this symptom. The patients felt better quickly and, in terms of biochemical parameters, we found quick improvements in lactate dehydrogenase levels as well.

H&O What were the next steps after this initial study?

AH The pilot study was initially planned to be 12 weeks in duration, but at the end of the 12 weeks, all patients wanted to continue with eculizumab therapy. The extension phase of the trial commenced and is still continuing with 10 of the 11 patients. Patients have experienced a dramatic improvement in their quality of life and generally prefer 2 weekly infusions of eculizumab rather than their previous treatment, which might have been, for example, 4 weekly blood transfusions. Some patients have become transfusion-independent.

H&O What side effects are associated with eculizumab?

AH The side effects associated with eculizumab have been very minimal. One patient in the pilot study had a reaction to the initial infusion, consisting of shivering and fever. She required observation only and has tolerated every infusion after that extremely well with no further reactions; she is still on the drug more than 3 years later. No other patients experienced any infusion-related reactions. The only minor side effect has been headache after the first or second infusions in some of the patients, but otherwise toxicity has not been a problem. There is a theoretical risk of meningitis, but this has not been a major issue to date.

H&O Is there any sense about whether eculizumab treats all patients equally or are there variations between patients that might change the efficacy of the agent?

AH We found that patients who have the classic purely hemolytic PNH respond better to the drug than those who have a hypoplastic bone marrow. The reason for this difference is simply that marrow failure can sometimes contribute more significantly than hemolysis to anemia, in which case, although the hemolysis will be blocked by eculizumab, marrow failure will most likely still lead to anemia and therefore continuing transfusions will be required. Certainly any patient with classical PNH who is hemolyzing frequently is a good candidate for eculizumab therapy. This agent would also be appropriate for patients with biochemical and clinical evidence of hemolysis because their quality of life can be improved.

H&O What is the clinical trial status of eculizumab?

AH Two phase III trials opened worldwide in the last year: TRIUMPH and SHEPHERD. The first opened about a year ago and the second opened earlier this year. The trials are now closed to recruitment, and we are eagerly awaiting the results. If the results of this trial are positive, I would expect that applications for product licenses would be made relatively quickly.

H&O Does eculizumab appear to have any effect on thrombosis, the most significant morbidity associated with PNH?

AH Although at the moment we have no evidence that eculizumab will prevent thrombosis, it is reasonable to hypothesize that thrombosis may be prevented by this terminal complement blockade through its mechanism of action. Theories for the causes of thrombosis in PNH include activation of PNH platelets by complement attack and nitric oxide depletion through free hemoglobin scavenging. Eculizumab should prevent both consequences. This possibility will be very interesting to examine in the future. In the pilot study there was no evidence of thromboses in any of the patients.

Suggested Reading

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〈 1136 〉 PACKAGING—UNIT-OF-USE

INTRODUCTION

This chapter provides guidance in the use and application of unit-of-use packaging and is intended for use by drug manufacturers, repackagers, and pharmacists. Suppliers of packages and packaging components may find the information useful, as well.

The *General Notices* defines a unit-of-use container as one that contains a specific quantity of a drug product that is intended to be dispensed as such without further modification except for the addition of appropriate labeling.

Unit-of-use packaging, when provided by the manufacturer, offers some of the following attractive advantages. (1) A dosage form can be dispensed to a patient in the manufacturer's original container, a practice that recognizes that the suitability of the container has been established on the basis of the manufacturer's stability studies. (2) The counting and repackaging of dosage units in the pharmacy is eliminated, thereby reducing the possibility of human error. (3) The pharmacist is able to affix the label for the patient onto the unit-of-use package and is free to use the manufacturer's expiration date as the beyond-use date. (4) The number of dosage units in a single unit-of-use package may be determined on a case-by-case basis. (5) Patient compliance is improved. (6) The unit-of use package can protect against countertfeiting because traceability of product is ensured through bar coding techniques and NDC numbers.

Unit-of-use packaging, when provided by repackagers, offers the same attractive advantages as those offered by the manufacturer. However, unit-of-use repackagers should conform to all requirements as presented in *Good Repackaging Practices* 〈 1178 〉. There are a number of reasons why repackagers produce unit-of-use packaging: for example, (1) requests from institutions, (2) better inventory control, (3) reduced dispensing times, and (4) variations in some drug therapies.

The packaging of a unit-of-use system may be a multiple container or a single-unit container. A unit-of-use system may contain a drug product in a liquid, semisolid, or solid dosage form (see also *FDA Guidance for Industry on Container Closure Systems for Packaging Human Drugs and Biologics*). [NOTE—The terms "unit-of-use package" and "unit-of-use container" may be used interchangeably.]

The Poison Prevention Packaging Act (PPPA) of 1970 requires in certain cases the use of special packaging—child-resistant and senior-friendly. Child-resistant packaging protects children from serious injury or illness resulting from ingesting or handling hazardous products including drugs.

Because drugs packaged in unit-of-use packaging are intended to be dispensed to the consumer without repackaging by the pharmacist, the manufacturer or repackager is responsible for the special packaging of PPPA-regulated substances in unit-of-use containers (l6 CFR 1701.1).

TYPES OF CONTAINERS FOR UNIT-OF-USE

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Unit-of-use containers are required to be child-resistant if they are intended to be dispensed directly to the patient pursuant to a prescription. Unit-of-use packaging intended for institutional or hospital use may or may not be required to be child-resistant. Unit-of-use containers that are child-resistant single-unit containers include supported blisters, such as separate, peel, push, and tear notch, and enclosed or in-card blisters, such as pull tabs and slide packs. Blister packaging is discussed in the general chapter *Packaging Practice—Repackaging a Single Solid Oral Drug Product into a Unit-Dose Container* ⟨ 1146 ⟩. Unit-of-use containers that are multiple-unit containers include glass and plastic containers.

Single-Unit Container

A single-unit container is one that is designed to hold a quantity of drug product intended for administration as a single dose or a single finished device intended for use promptly after the container is opened. Preferably, the immediate container and/or the outer container or protective packaging shall be so designed as to show any evidence of tampering with the contents. Each single-unit container shall be labeled to indicate the identity, quantity, and/or strength, name of the manufacturer, lot number, and expiration date of the article.

Unit-Dose Container

A unit-dose container is a single-unit container for articles intended for administration by other than the parenteral route as a single dose, directly from the container.

Single-Dose Container

A single-dose container is a single-unit container for articles intended for parenteral administration only. It is labeled as such.

Multiple-Unit Container

A multiple-unit container is a container that permits withdrawal of successive portions of the contents without changing the strength, quality, or purity of the remaining portion.

PACKAGING FABRICATION MATERIALS

Packaging fabrication materials include substances used to manufacture packaging containers such as glass, plastics (including high-density polyethylene (HDPE), low-density polyethylene (LDPE), polyethylene terephthalate, polyethylene terephthalate G and polypropylene (PP), other resins, and other materials as listed in the general test chapter *Containers* ⟨ 661 ⟩ and in the *FDA Guidance for Industry on Container Closure Systems for Packaging Human Drugs and Biologics*.

Glass

Any glass packaging material used in the immediate container should meet the glass test requirements for *Limits for Glass Types* and *Chemical Resistance—Glass Containers: Powdered Glass Test, Water Attack at 121°*, and *Arsenic* under general test chapter *Containers* ⟨ 661 ⟩.

Plastic

Any plastic packaging material used in the immediate container should meet the plastic test requirements for *Plastics* in the general test chapters *Containers* ⟨ 661 ⟩ and *Containers—Permeation* ⟨ 671 ⟩. Depending on the type of plastic packaging material used, the packaging material meets the requirements for *Biological Tests—Plastics and Other Polymers, Physicochemical Tests—Plastics, Polyethylene Containers, Polyethylene Terephthalate Bottles and Polyethylene Terephthalate G Bottles,* and *Polypropylene Containers* under general test chapter *Containers* ⟨ 661 ⟩.

The test for moisture vapor transmission may be carried out as described in the general test chapter *Containers—Permeation* ⟨ 671 ⟩ for multiple-unit and unit-dose containers.

PACKAGING CLOSURE TYPES

Reclosables and nonreclosables may be used for solid, semisolid, and liquid dosage forms. Both must be packaged in compliance with the 16 CFR 1700.15 standards.

Reclosables

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Reclosables are containers with suitable closures that may incorporate tamper evidence and child-resistance capabilities. Reclosables may be used for glass or plastic containers.

Nonreclosables

Nonreclosables are containers with closures that are nonreclosable, such as blisters, sachets, strips, and other single-unit containers. Nonreclosables may include packs such as cold-formed foil blisters, foil strip packs, and PVC/Aclar combining multilayer materials that are thermo-formed or cold-formed foil blisters (see *Packaging Practice—Repackaging a Single Solid Oral Drug Product into a Unit-Dose Container* ⟨ 1146 ⟩). Nonreclosables may be child resistant depending on the intended use and place of use. Household nonreclosables are subject to the PPPA as defined in 16 CFR 1700.14. However, because of some unit-dose designs, not all unit-dose packages comply with the PPPA.

LABELING

The unit-of-use containers are labeled to include expiration dates, the manufacturer's lot number, the NDC designation, and bar codes as provided in the *Labeling* section of the *General Notices and Requirements* under *Preservation, Packaging, Storage, and Labeling* and in *Good Repackaging Practices* ⟨ 1178 ⟩. Some of the advantages of having bar codes on the label include reduced medication errors, improved inventory control, and improved access to medication identity. The labeling covers information placed in the container by the manufacturer (see *General Notices and Requirements*). Acceptable labeling can range from the full labeling as for multiple-unit containers to an abbreviated labeling when the container is too small to include all the text. Full labeling may also be provided on the carton if it is not present on the immediate container.

REPACKAGING AND REPROCESSING

Unit-of-use containers are reprocessed or repackaged as instructed by the manufacturer or as directed in the general test chapter *Containers* ⟨ 661 ⟩ or in the general information chapter *Packaging Practice—Repackaging a Single Solid Oral Drug Product into a Unit-Dose Container* ⟨ 1146 ⟩. A unit-of-use package that is a blister package may not be reprocessed by a pharmacist once it has been deblistered from a unit-dose container (see *General Notices and Requirements* for application of the appropriate beyond-use date for a multiple-unit or unit-dose container). Deblistering is the process of removing medication from a blister-type container. However, under current Good Manufacturing Practices (cGMPs) and tight quality controls, the manufacturer or contract repackager may repackage and reprocess unit-of-use containers.

INFORMATION FROM MANUFACTURERS

The manufacturer should provide appropriate stability information that can be used to determine appropriate labeling, storage, and shipping statements that will properly inform patients and practitioners. The manufacturer may make other assurances based on product information on packaging and distribution arrangements. In the event that a product is not to be repackaged, the manufacturer may so state in the labeling. The manufacturer also includes labeling and information suitable for optimal handling by the practitioner and the patient. The labeling and information should be bar coded to eliminate medication error and promote medication traceability.

RESPONSIBILITY OF THE DISPENSER

Labeling

The labeling on a unit-of-use container also includes a label added at the dispensing stage by the pharmacist. Prior to dispensing the unit-of-use package, the dispenser shall add label(s) that provide the following information:

1. the name of the patient;
2. the name and strength, the directions for use as prescribed by a doctor or health-care provider, and the name of the prescriber; and
3. any storage instruction, beyond-use date, and other information as deemed appropriate by federal and state laws.

In the pharmacy setting, pharmacists are encouraged to use bar codes, in conjunction with computerized prescription orders, to confirm that the right drug is being dispensed to the right patient. Bar coding would minimize errors and create opportunity for medication traceability and accountability.

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Information to Patient

Patients must be given information that applies to the specific prescription being dispensed.

QUALITY CONTROL OF PACKAGING SYSTEM

The packaging system shall meet the general considerations for system suitability, protection, safety, and performance characteristics as described in *FDA Guidance for Industry on Container Closure Systems for Packaging Human Drugs and Biologics*, in the general test chapters *Containers* ⟨661⟩ and *Containers—Permeation* ⟨671⟩, and in the general information chapter *Packaging Practice—Repackaging a Single Solid Oral Drug Product into a Unit-Dose Container* ⟨1146⟩.

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D9

AN - NLM1411724
DT - Case Reports, Journal Article
TI - Recurrence of **neisserial meningococcemia** due to deficiency of terminal complement component.
AU - Morris J T; Kelly W J
PUB - Southern medical journal
- UNITED STATES
- Oct 1992
ORD - 1992-10-00
LNKD - **pubmed:1411724**
IRN - ISSN 0038-4348 *(Print)*
VOL - 85
NR - 10
PG - 1030 - 1031
LA - eng
IW - Adult; Bacteremia: diagnosis, drug therapy, etiology(#); Complement C6: deficiency(#); Humans; Immunologic Deficiency Syndromes: complications(#); Male; Meningococcal Infections: diagnosis, drug therapy, etiology (#); **Neisseria meningitidis**(#); Penicillin G: therapeutic use; Recurrence
AW - Complement C6; Penicillin G
AB - Recurrent infections with **Neisseria meningitidis** are attributed to deficiencies of terminal complement components. The serotype most commonly responsible for recurrent N meningitidis infections is serotype Y. We have reported a case of recurrent meningitis due to N meningitidis in a patient who was found to be deficient in the sixth component of complement. **Complement deficiencies** should be considered in any patient with recurrent infections caused by N meningitidis.

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AN - NLM15328683
DT - Case Reports, Journal Article, Review
TI - Complement C7 deficiency presenting as recurrent aseptic meningitis.
AU - Corvini Michael; Randolph Christopher; Aronin Steven I
PUB - Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology
- United States
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AW - Complement C7; Complement Membrane Attack Complex
AB - *BACKGROUND*
Complement deficiency states are rare inherited disorders that may predispose affected individuals to angioedema, collagen vascular disease, or infection due to encapsulated organisms, especially **Neisseria meningitidis**

- *OBJECTIVES*
To report the case of a 36-year-old man of Irish descent with recurrent culture-negative neutrophilic meningitis, to offer potential reasons for the inability to recover a causative pathogen, and to review the genetics and prevalence of **complement deficiency** states, the methods of screening for such deficiencies, the features of meningococcal infection as they relate to such deficiencies, and management strategies for clinicians caring for patients with such deficiencies.

- *METHODS*
The patient presented in 1988 and again in 2002 with culture-negative neutrophilic meningitis. His second episode was characterized by a rash suggestive of meningococcal infection, prompting immunologic evaluation.

- *RESULTS*
Immunologic evaluation revealed an undetectable CH50 level. Levels of C1, C2, and C5 through C9 were normal except for C7, which was undetectable. Further testing revealed that the patient's sister was also C7 deficient.

- *CONCLUSIONS*
Complement component deficiencies are relatively rare; individuals with collagen vascular disease and systemic neisserial infection should be screened using either the CH50 or the APH-50 assay. Key to the management of a late-complement component-deficient host is counseling, education about meningococcal infection, and discussions about the potential benefits of chemoprophylaxis and immunoprophylaxis. The ability to detect the bacterial cause of meningitis in such patients is organism dependent and may be influenced by factors such as cerebrospinal fluid bacterial concentration and previous antibiotic drug exposure.

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VACCINE AGAINST GROUP B NEISSERIA MENINGITIDIS: PROTECTION TRIAL AND MASS VACCINATION RESULTS IN CUBA

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SUMMARY

The Cuban vaccine, first in the world with proven efficacy against group B-caused disease, is based on outer membrane proteins from B meningococci capable of inducing long-lasting and high-titered bactericidal antibodies in humans. This bactericidal activity has a wide spectrum against all pathogenic group B *Neisseria meningitidis* tested.

A randomized, double-blind controlled trial of the vaccine efficacy was performed during 1987-1989 with 106 000 10-14 years old students from 197 boarding schools in seven provinces.

The efficacy obtained was 83% (χ^2, $p<0.002$; Fischer exact, $p<0.001$). In a second field trial including 133 600 persons from 5 months to 24 years of age in Ciego de Avila province (30 cases/10^5 inhabitants, the highest incidence rate in Cuba) by comparing vaccinated and non-vaccinated population after 2.5 years of observation and careful follow-up, the efficacy and safety was confirmed.

Because of these results and because of the very low reactogenicity of the vaccine, the Ministry of Public Health took the advice of the Scientific Council to vaccinate all children between 3 months and 6 years of age in the most affected provinces. No severe or long lasting reactions to the vaccine were observed after the millions of doses administered. The efficacy of vaccination varied in the provinces between 83% and 94%, among age groups ranging from 3 months and 20 years.

After 3 years of massive application no severe reactions occurred and one of the most severe epidemics has been practically eradicated.

Key words: Evaluation studies; Neisseria meningitidis; Randomized controlled trials; Vaccines

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INTRODUCTION

Since 1976, meningococcal disease constitutes a serious health problem in Cuba due to the significant rise in incidence of this disease and its high lethality.

The causal agents at beginning of the outbreak were predominantly group C meningococci and in second place group B meningococci; after the mass vaccination campaign conducted in 1979 using the polysaccharide Merieux A + C, group C caused meningococcal disease was practically eradicated.

In the late 70s and early 80s, a significant incidence increase took place, virtually caused by B meningococci of 4:P1.15 (95%), 15:P1.15 (3%) and 15:P1.16 (1%) serotypes and subtypes.

The peak epidemic year was 1983, although the situation differed from province to province. Higher incidence rates were observed in 1985 in children younger than 6 years (50 cases per 10^5 inhabitants) and among teenagers (20 cases per 10^5). The most affected teenagers were those attending secondary boarding schools in the central provinces of Cuba (57,7 cases per 10^5 students).

B-meningococcal disease has been the most serious infection health hazard of the last two decades in Cuba.

Repeated testing with different vaccine candicates in the United States, Europe, the Soviet Union, and South Africa, revealed many deficiencies among these; a weak, very variable and short-lasting (5-7 months) bactericidal activity; beyond 5-7 months after immunization the concentration of specific antibodies of IgG class, directed against important exposed proteins and other antigens of the bacterial envelope, was very low. In addition, weak or absent anamnestic response to a booster dose after 8 to 12 months of primary immunization was observed as one of the most critical failures.

Thus, taking into consideration the world research state in the B meningococcus vaccine development and our situation with the disease, decision was taken to obtain in short time a good candidate vaccine.

Among different vaccine formulations one was selected for which carefully conducted preclinical and phase I and phase II clinical studies revealed innocuousness, safety and very low reactogenicity, long-lasting bactericidal and specific antibody titers of a broad spectrum against different pathogenic serotypes and subtypes. On the basis of these promising serological efficacy results, this vaccine has been used in a double-blind placebo-vaccine field trials for epidemiological efficacy evaluation.

MATERIALS AND METHODS

Base-line studies

Base-line data on epidemiology of the disease and diagnostic laboratory capability were collected and evaluated during a three-year period in the provinces, before the trial-started (1984-87). Laboratory procedures, case management protocols, and the system of coordinating information from schools, hospitals, provincial, municipal and national epidemiologists, and laboratories have been carefully reviewed, evaluated and when needed established.

Case ascertainment and case definitions were established as well as the isolates and samples transport-conservation-study system.

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Vaccine trial design

The trial was a randomized, placebo-controlled, double blind trial of vaccine efficacy. The protection trial was performed among teen-agers attending secondary boarding schools. The target group ranged, from 11 to 16 years of age and was distributed in 197 schools and 7 provinces with the highest incidence rates in this age group (40 cases per 10^5 students at the starting point of the trial).

Sample size was calculated according to the level of efficacy to be detected at 75% or greater, with less than a 0.05 type I error ($\alpha = 0.05$) and a power of 80% ($\beta = 0.2$, taking into account an attack rate of the disease of 40 cases per 10^5 students and a follow-up period of 1-3 year. With this incidence rate at the start of the trial and considering the other statistical factors, 106 251 students for placebo and vaccine groups were calculated to be needed in using schools as randomization units. Each school with approx. 540 students constitutes a homogeneous unit, either completely vaccinated or completely placebo inoculated.

A list of schools was assembled and stratified by province according to incidence of the disease during the last three years, to assure homogeneous distribution of exposure to risk of disease in receiving placebo or vaccine. Afterwards, placebo or vaccine were randomly allocated in each school within every stratus.

To assure blinding, lot identification was performed with randomly generated numbers having a secret master code list. Ten different numbers for placebo and ten for vaccine were used.

The vaccine

The vaccine components are purified proteins from the outer-membrane of group B meningococci enriched with proteins from the high molecular weight proteins complex (65-95 KD) having the greatest capacity of inducing long-lasting bactericidal antibodies. Some of the purified proteins, together with a good controlled proportion of phospholipide and lipopolysaccharide (LPS) form a proteoliposom, and the added amounts of High Molecular + Protein-Complex is non covalently conjugated or attached to the proteoliposome surface. In addition, the whole protein complex is conjugated non covalently, 1:1 proportion with C polysaccharide and finally the whole complex is adsorbed to aluminium hydroxide gel with a controlled particle size.

Vaccine formulation

Each dose contains:

Outer membrane proteins from B meningococci	50 mcg
Purified polysaccharide form C meningococci	50 mcg
Aluminium hydroxide gel	2 mg
Sodium thiomersalate	0.01 mg
PBS csp	0.5 ml
LPS	1%

The commercial name of this preparation is VA-MENGOC-BC®, a trade mark of Institute Finlay, Habana, Cuba.

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Control vaccine (Placebo)

The control vaccine was identical in appearance to the tested vaccine and consisted of a saline placebo containing the adjuvant aluminium hydroxide.

Vaccination, sampling and evaluation

A two 0.5 ml deltoid intramuscular dose with 6 to 8 weeks interval was the vaccination schedule established.

In each province, 300 students randomly chosen were used for immunogenicity and carrier state studies. The samples for these studies were taken immediately before applying the first dose and 4 weeks after the second one. Additional blood and exudate samples were taken every six months after vaccination. ELISA and bactericidal studies were performed as described elsewhere (1).

Only cases with the following criteria were used for efficacy evaluation: confirmed cases are only those with clinical illness from which a B meningococcus was isolated by culture or those with clinical illness with Gram-negative diplococci directly observed and where a latex or ELISA confirmed B antigen was found.

The standard forms for data collection, previously field-tested, were submitted to the Ministry of Public Health and stored in the Efficacy Trial Data Base Computer System.

SAS-PC version 6.03 and EPI-INFO were used for data analysis and study monitoring, relative risk calculation for vaccine and placebo groups with schools as randomization units, individual or cluster approaches. The comparisons between placebo and vaccinees regarding ELISA and bactericidal titer distributions were performed with the help of a computing program having common non-parametric tests, Wilcoxon Rank-Sum Test, t-Test, percentile distribution and binomial distribution analysis of probabilities and the statistical analysis significance tests-Chi-square and Fischer Exact.

RESULTS AND DISCUSSION

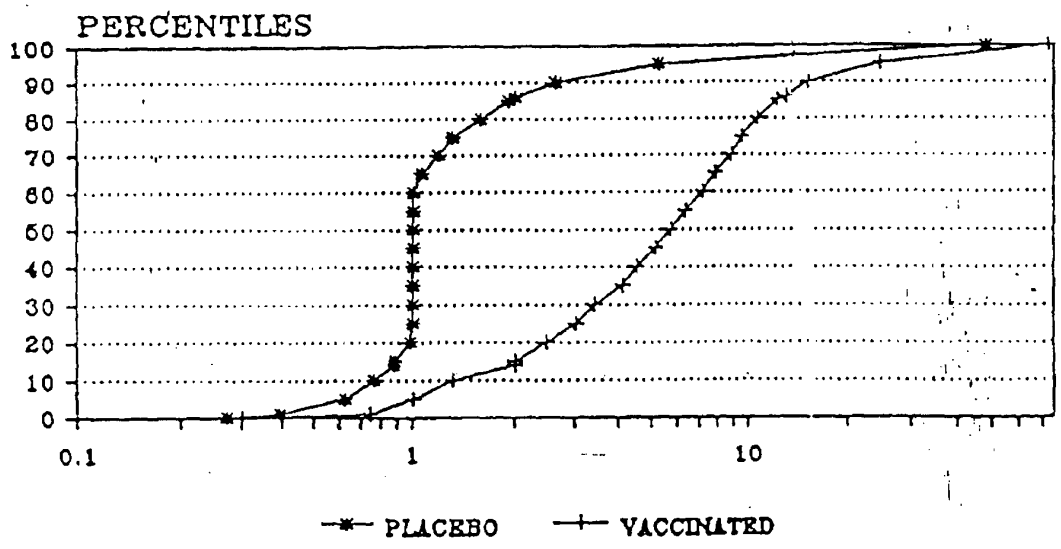


Figure 1
Cumulative distribution of the ratio ELISA2/ELISA1 four weeks after vaccination

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The study of the meningococcus carrier state shows no significant difference between placebo-injected controls and vaccinees when the sample was taken four weeks after vaccination. Six months after vaccination, a significant lowering in carrier state in vaccinees was observed, but more careful assessment of variations and mechanisms must be performed in the future for conclusive statements.

In the vaccine group, an 88% of seroconversion (E2/E1) was detected using the ELISA method; in percentile 50 (median of the group), a six-fold increase was observed. On the other hand, in the placebo group, percentile 80 shows just one as E2/E1 ratio and only 14% of this group shows two-fold increase (correlating quite well with carrier state). Comparing the cumulative distribution of the E2/E1 ratio in placebo and vaccine four weeks after vaccination, a significant difference with $p < 0.001$ is seen..(Fig. 1).

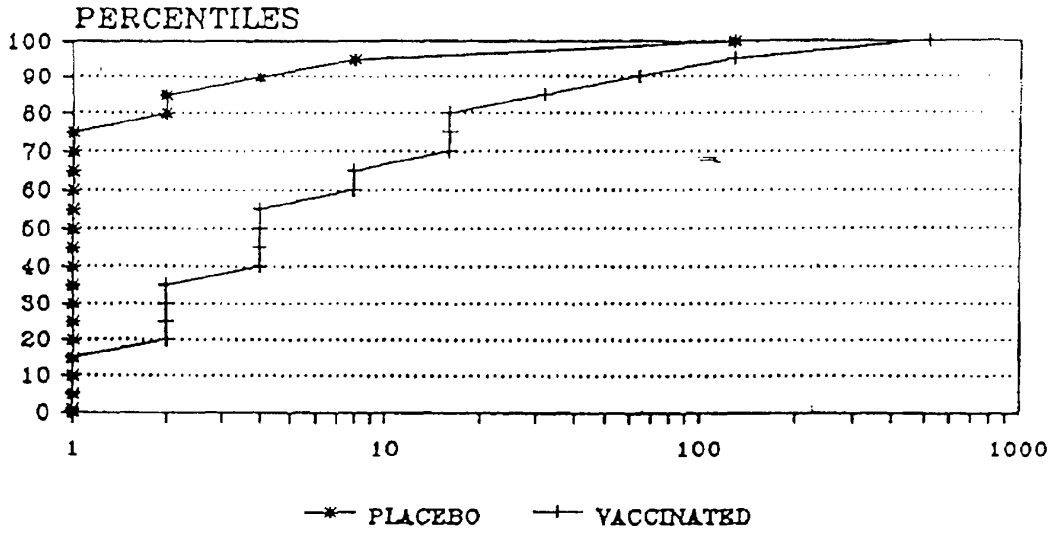


Figure 2
Cumulative distribution of the ratio BACTE2.Titer/BACTE1.Titer four weeks after vaccination

Seroconversion regarding bactericidal activity was detected in 85% of the vaccine group in which percentil 50 (median) shows a four-fold titer increase (Fig. 2). The difference with respect to the placebo group was highly significant, $p<0.001$. Only percentil 85 shows two-fold increase in this group caused by normal contacts with meningococci in the population. The cumulative distribution of the ratio B2/B1 comparing placebo and vaccine group shows statistical significance.

This study lasted 16 months, during which 25 culture and latex positive confirmed cases occurred in the study population, four in the vaccine group and 21 in the placebo group. The timing of cumulative case occurrence among placebo and vaccine group, as seen further on, constitutes a good graphic demonstration of the vaccine effect (Fig.3). Case occurrence was distributed per school as indenpendent cases and fulfilling the criteria of «case definition» needed in order to be included in the efficacy calculation.

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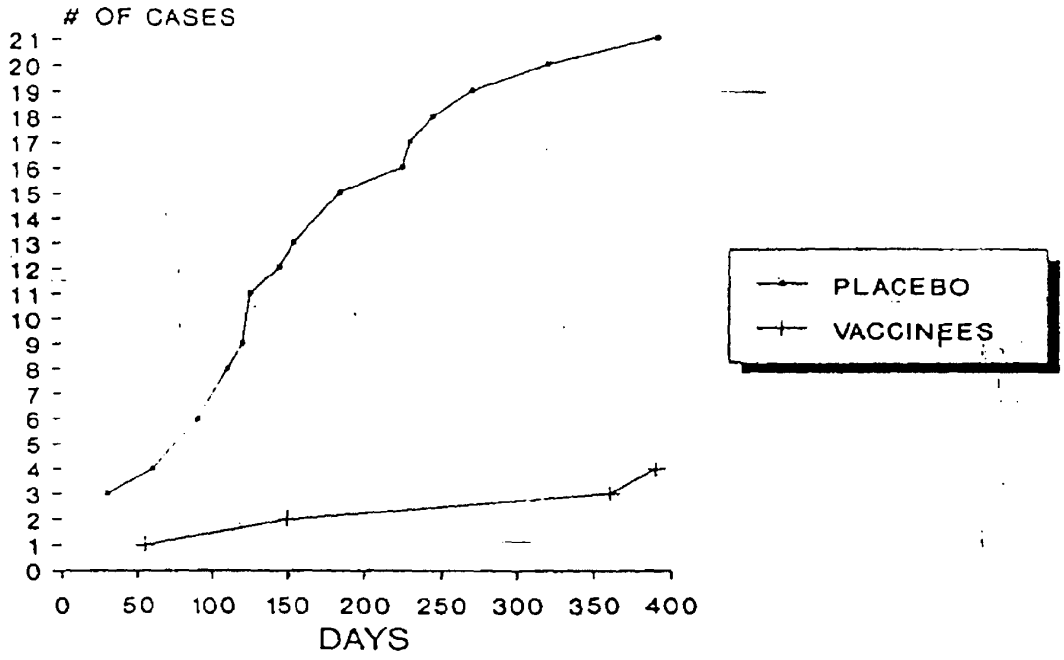


Figure 3
Timing of cumulative case occurrence

Further observation up to 24 months revealed the occurrence of five more bacteriologically confirmed cases, all in the placebo group and none among the vaccinees though not included in the following efficacy calculation:

Efficacy according to conglomerate approach

	Vaccine	Placebo	Total
Total of schools involved	99	98	197
Schools with cases	3	17	20
Total of cases	4	21	25

Analysis of Single Table
Relative risk-0.17 (0.05<RR <0.58)
Taylor series 95% confidence limits for RR

	Chi-squares	p-values
Uncorrected	11.07	0.0008791
Mantel-Haenszel	11.01	0.0009061
Yates corrected	9.55	0.0019968

Resultant efficacy: 83%
Confidence interval: 95-42%

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Efficacy according to individual approach

Group	Cases	Not ill	Total
Vaccinee	4	52962	52966
Placebo	21	53264	53285
Total	25	106226	106251

Analysis of Single Table
Relative risk-0.19 (0.07< RR<0.58)
Taylor series 95% confidence limits for RR

	Chi-squares	p-values
Uncorrected	11.46	0.0007107
Mantel-Haenszel	11.46	0.0007108
Yates corrected	10.15	0.0014457

Resultant efficacy: 81%
Confidence interval: 93-44%

Vaccine impact is also shown by demonstrating the significant reduction in case-occurrence probability in vaccine schools in comparison with placebo treated schools. Immediately before the starting point of the field trial the probability of one or more cases per school in the study was calculated (Po = 0,192). After 1.3 year of follow-up period 17 cases had occurred in 98 placebo schools. The probability of case occurrence was 0.173469 (Pp). The simultaneous case occurrence in vaccine schools was 3 in 99 schools.

Binomial distribution

	N = 99	P.1734693
X	P(X)	Cumulative probability
3	.00001	.00001
4	.00005	.00006
5	.00019	.00025
6	.00062	.00086
E(X) = 17.173346	std dev = 3.76754	variance = 14.19439

A way to evaluate the impact of the vaccine is by calculating under the hypothesis that Po = 0.192 (previous value) the probability of the happening in placebo and vaccine groups. The probability associated with the occurrence of three or less schools with at least one case in a set of 99 (vaccine groups) and 17 or less of a set of 98 (placebo groups) are 0.00001 and 0.38 respectively. These results suggest that p in the vaccine group is

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significantly less than Po, but not so in the placebo groups. Another approach is using the placebo value of p in determining the probability of that happening in the vaccine group. Having p the value of 0.173 (17/98), then, P (X3)0.00001. This value of p is significantly less than 0.173.

The difference in case occurrence among placebo and vaccine groups was statistically significant, considering the cases individually or effecting a conglomerate (school distribution of cases). The vaccine proved to be effective in the study population in 81% (individual treatment of cases) or 83% (conglomerate treatment), and the confidence interval from 44 to 93% or 42 to 95% respectively.

Further follow-up studies

Furthermore, a good correlation between serological evolution and protection as well as the low reactogenicity and safety of the vaccine was confirmed.

The comparison of ELISA and bactericidal ratios E4/E1 and B4/B1 respectively constitutes an objective proof of duration of the vaccine protective capacity. E4 and B4 represent ELISA and bactericidal values one year after vaccination (Figs. 4 & 5).

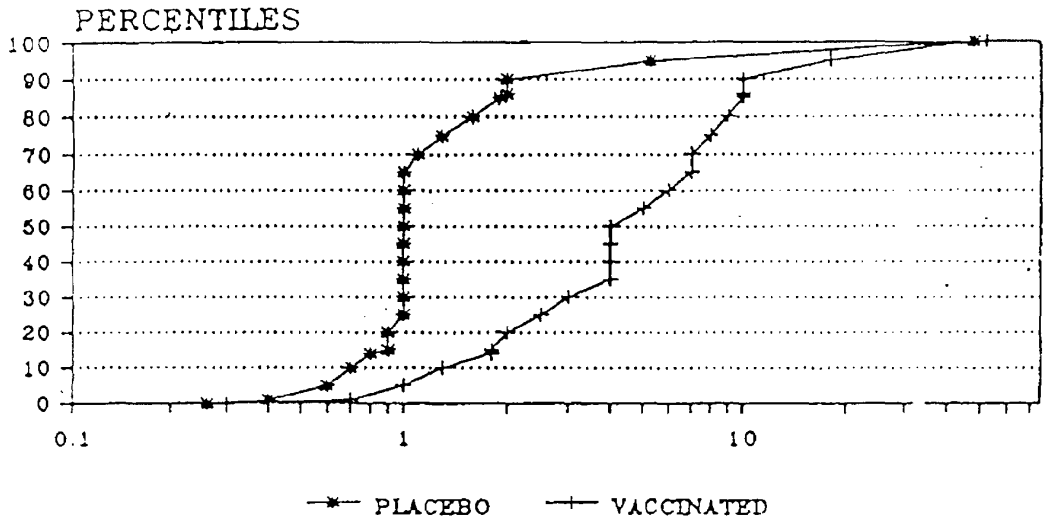


Figure 4
Cumulative distribution of the ratio ELISA4/ELISA1 one year after vaccination

The anamnestic antibody response was studied in army recruits, in parallel. A booster dose was tested in one group after one year and another group after 18 months. Before boostering (12 to 18 months) blood plasma from the vaccine group of the parallel study was collected, and the IgG fraction was purified and tested by ELISA and bactericidal activity (Figs. 6 and 7).

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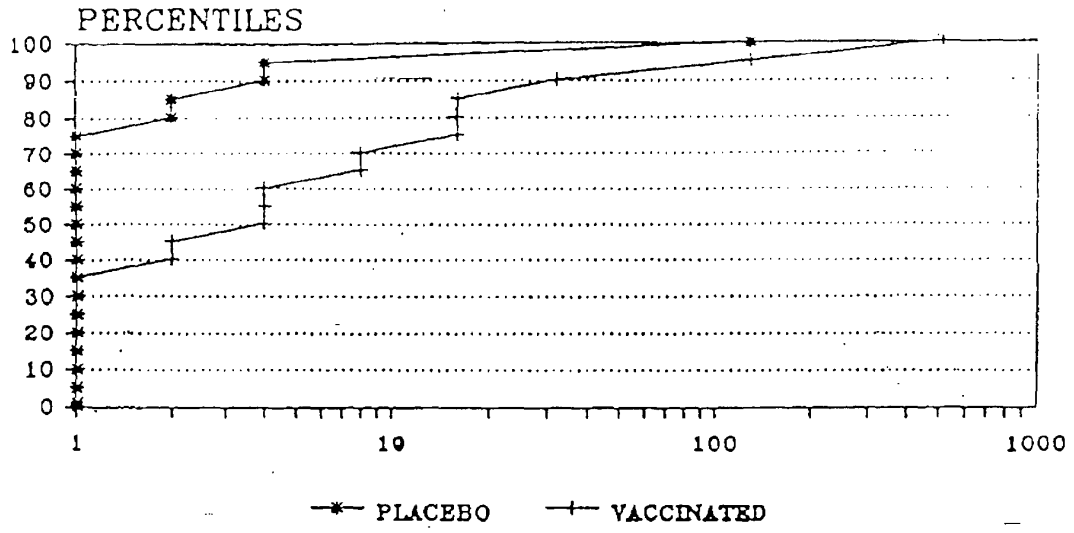


Figure 5
Cumulative distribution of the ratio BACTE4.Titer/BACTE1.Titer one year after vaccination

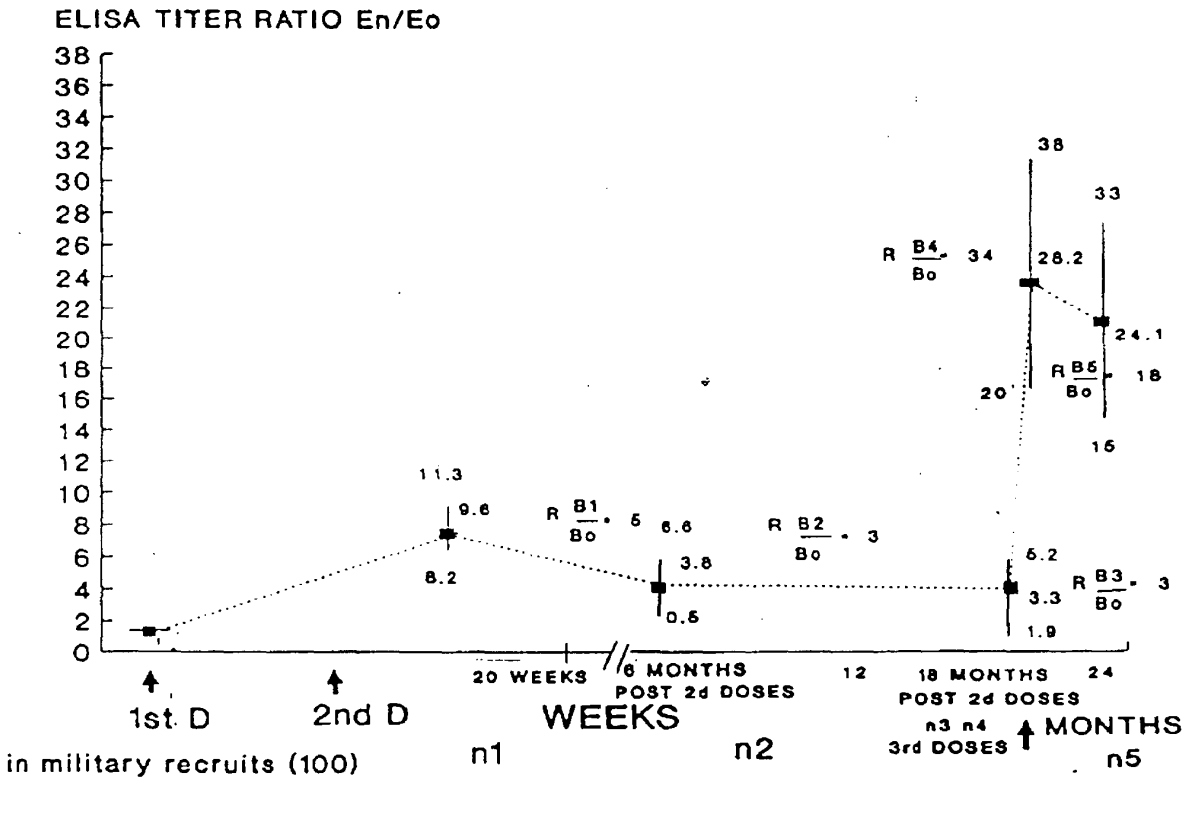


Figure 6
Increasing bactericidal and specific antibodies with a booster

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The results of these tests indicated long-lasting bactericidal and specific antibodies against some of the most frequent pathogenic B serotypes. After boostering the specific antibody increased by more than 5 times and the bactericidal titer increased more than 10 times, showing an excellent anamnestic specific response.

Summary of mass vaccination results

Taking into consideration the high incidence of meningococcal disease among children up to six years of age occurring in Cuba and the satisfactory reactogenicity and efficacy studies with the Antimeningococcal BC Vaccine «VA-MENGOC-BC®» evaluated during the last years, a mass vaccination campaign was organized, starting with this age-group (1989-90). Table 1 shows the percentage of vaccination per province in the age-group below six years.

An independent group of the Tropical Medicine Institute «Pedro Kouri» of Ministry of Public Health evaluated in a carefully conducted study, the effectivity of the mass vaccination campaign. Table 2 shows the summary of efficacy, in general reaching 92.5%.

One very clear example of the vaccine impact was observed in the province Havana City, Fig. 8, where the incidence rate among children below six years rose constantly (1986 - 27.5/10^5, 1987 - 30/10^5, 1988 - 42.5/10^5) and after the vaccination the strong epidemic wave was stopped (1989 - 15/10^5, 1990 - 3.8/10^5, 1991 - 1/10^5) and among vaccinees less than 0.08/10^5).

Actually in Cuba the population between 3 months and 20 years is vaccinated; the VA-MENGOC-BC® vaccine is incorporated to the infancy vaccine scheme (for 3 months children).

Also the vaccination of other risk-groups is practically concluded; elderly homes, military camps, big schools, industries, *etc.*

In a very short time the meningococcal disease will be completely controlled.

Reference

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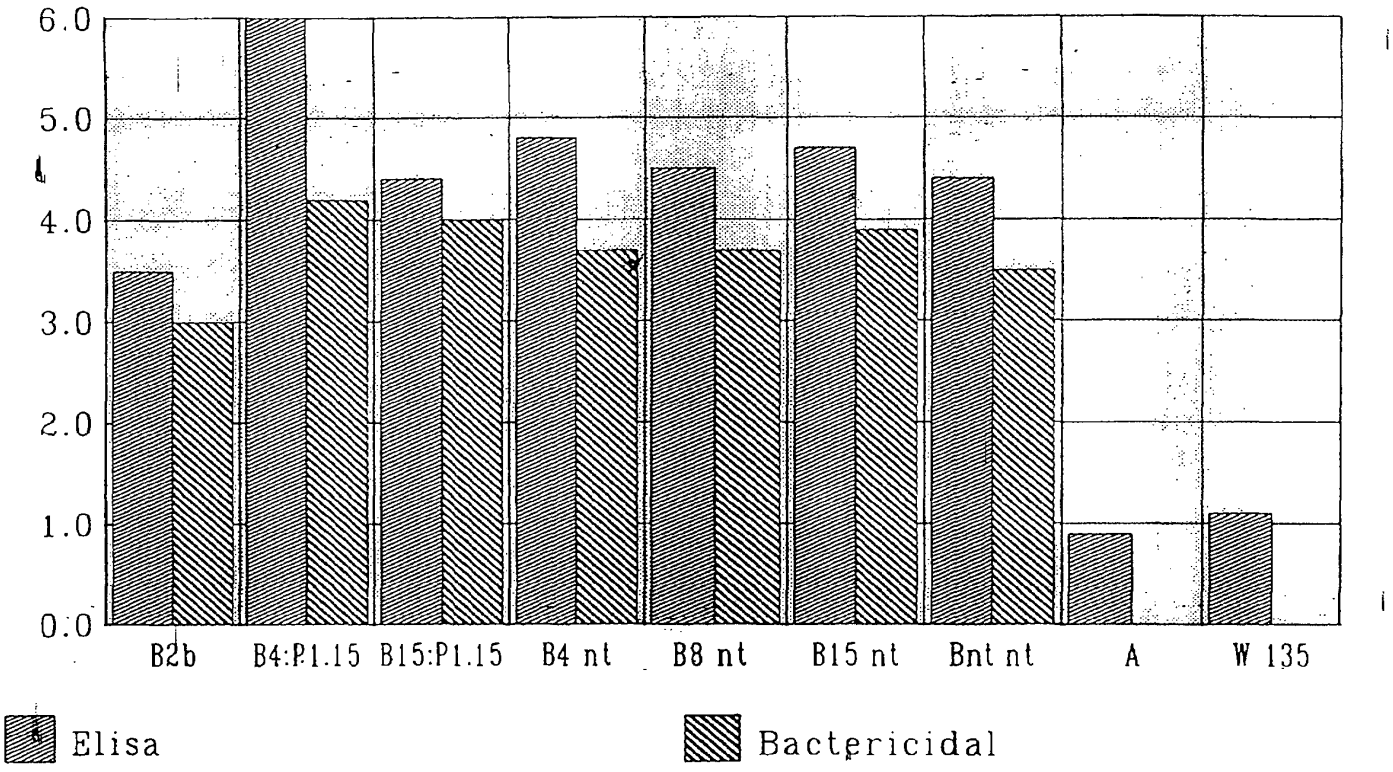


Figure 7
Specific and bactericidal antibody titer increases maintained up to 18 months after vaccination in military recruits against different pathogenic serotypes

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Table 1
Percentage of vaccination per province in age group < 6 years

Province	Population	Vaccinees	%
PR	67 500	56 623	83.8
CH	182 400	161 670	88.6
MT	53 000	48 815	92.1
VC	70 000	61 047	87.2
CF	33 800	32 094	95.0
SS	37 700	31 935	84.7
CM	73 500	61 917	84.2
LT	53 100	43 808	82.5
HO	102 900	91 191	88.6
GM	94 800	72 898	76.9
SC	116 600	100 820	86.5
GT	61 100	53 679	87.9
TOTAL	**1 013 900**	**866 148**	**85.4**
Source: Division of Epidemiology, Ministry of Public Health			

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Table 2
Relative risk and efficacy per province in age group < 6 years

Province	Relative Risk	Efficacy %
PR	9.37	89.3
CH	39.06	97.44
MT	23.33	88.57
VC	24.55	95.90
CF	23.00	95.68
SS	11.08	90.97
CM	7.48	86.60
LT	23.60	89.28
HO	97.49	98.97
GM	29.99	96.66
SC	6.84	85.47
GT	18.11	94.47
TOTAL	13.44	92.56

Source: Tropical Medicine Institute «PEDRO KOURI», Ministry of Public Health

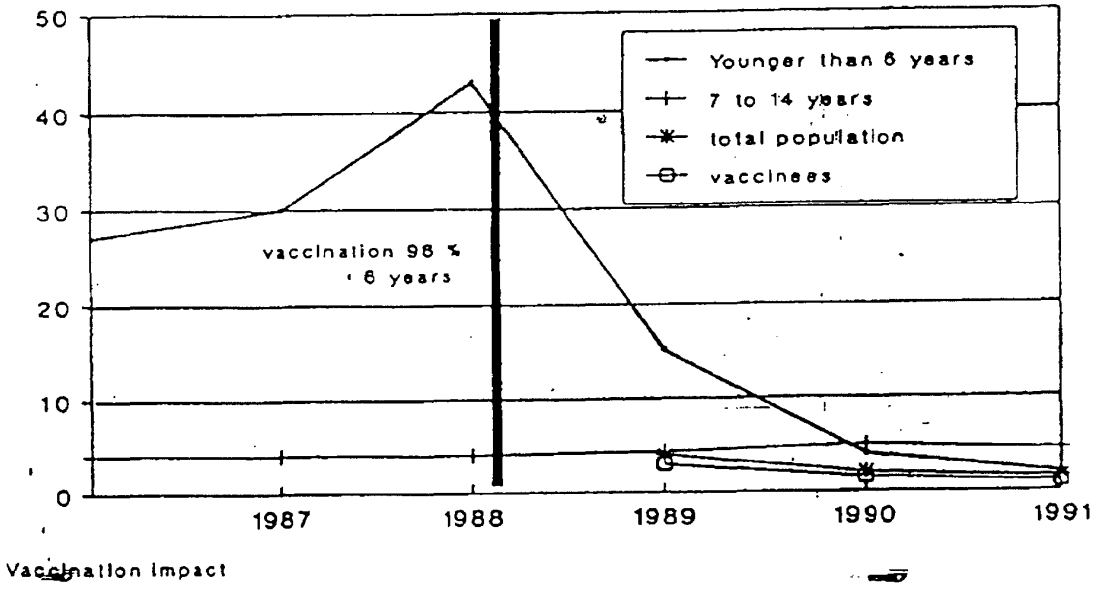


Figure 8
Meningococcal Disease in Havana city. indicence rate per 100 000

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DISCUSSION

Gotschlich:
To what do you attribute the broad specificity of the vaccine? What strains were tested for bactericidal effect, and what is the relative bactericidal effect against other strains?

Sierra:
All the results are related to the vaccine strain B:4:P1.15. We have also evaluated the spectrum of specific antibodies in ELISA and in bactericidal assay using different strains.

Gotschlich:
Could you just give us a list of the strains against which the bactericidal test was performed?

Sierra:
We have used B:4:P1.15, B:15:P1.15, B:4:NT, B:2a, B:2b, B:2c, B:8.

Gotschlich:
Could you try to speculate on the reason for the wide cross reactivity; which antigens do you think this is due to?

Sierra:
We think that this long lasting cross reactivity is due to the presence of high molecular weight proteins. There are six important proteins in this rank between 65 to 95 000 daltons. Two or three of these are iron-regulated proteins.

Gotschlich:
Which ones are iron-regulated?

Sierra:
One is a protein of 80 000 daltons, the other is very near to 90 000 daltons. We have now cloned, expressed, and purified some of these high molecular weight proteins, and we have characterized them. It is very difficult to obtain these in sufficient amount for characterization. We have demonstrated the importance of these proteins, especially for the immunological memory and for the cross reactivity.

Gotschlich:
There was yesterday a remarkably careful follow-up of patients that had been vaccinated, in terms of long term sequelae. Have you had the opportunity to investigate this in the same details as what was presented yesterday?

Sierra:
Yes, we studied it in Cuba very carefully. The first group of people was vaccinated in Cuba in 1985, and we have until now followed all the groups of the phase 1, phase 2, and phase 3 trials. We have no long-lasting sequelae at all. We have no disease caused and established through the vaccine. The most important reactions we have observed are the purpuric reactions. Very few cases have occurred, all over the country, especially in very small children. They were very short-lasting reactions. There were no sequelae and no neurologic diseases at all.

Peltola:
What do you mean by purpuric reactions? Do you mean petecchiae? If so, they usually are not very short-lived.

Sierra:
There are two types of reactions. One type of rash seems to be some type of allergy to some of the components and the other type of reaction consists of very small petecchiae. Both are short lasting. I think it is very important also to take into consideration the experience that we have in collaboration with Brazil in São Paulo. They have vaccinated nearly two and a half million children below six-seven years of age within in one week. They have experience in evaluating these side reactions.
They also observed a small number of these purpuric reactions or petecchiae. As far as I know, in Cuba or in Brazil we don't have long-lasting sequelae disease or severe reactions caused through the vaccine.

Griffiths:
You think that these iron-regulated proteins are important in your vaccine. Do you take any special measures to insure that they are expressed?

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Sierra:
I have not said that the iron regulated proteins are the only ones responsible for the efficacy. I have said that among the four most important high molecular weight proteins responsible for the long lasting bactericidal response, at least two proteins were related to iron metabolism.

Gotschlich:
The question was: Do you purposely limit iron for growth?

Sierra:
We have performed some changes in media regarding the concentration of some nutrients, but not especially iron limitation.

Wetzler:
Were there any non-volunteers in your study, and if there were, what was the incidence of disease in that group?

Sierra:
The incidence among people not volunteering in the study, in the same region or in the neighbourhood of these schools, has remained on the same level. In that age group we have studied this question intensively. We are going to publish all these results very soon.

Robbins:
I have two questions. The dose of the vaccine you mentioned was 50 micrograms. Was that based upon protein? And if it was based upon the protein, what was the total weight of all the material in the vaccine per dose? And the second question: what percentage of cases of bacterial meningitis were you unable to diagnose either by culture or by serological methods?

Sierra:
The vaccine composition is 50 micrograms of proteins per dose. Between 12 and 18% of these are proteins of the high molecular weight complex. We purify the outer membrane proteins, purify the proteoliposomes and we enrich these preparations with high molecular weight proteins. This is not simply a purification route. The composition is approx. 85% of the normal serotype proteins and approx. 15% of high molecular weight proteins. And we try to avoid the presence of class 4 and class 5 proteins in the vaccine because we have found a great variability in the presence and expression of these proteins in the strains circulating in our country, even before the P1.15 epidemic. We have a limit for tolerance of these proteins in the vaccine. We also avoid the B polysaccharide in the vaccine. We test the preparation chemically and also for the capacity to induce antibodies. We have found that the preparation is not able to induce significant anti-B antibodies.
To the second question, related to the incidence: we were not able to find the causal agent in three cases among vaccinees and in two cases of placebo. But after these we have applied different serological analytical methods. We have tested very carefully for the presence of antigens.
A case of meningococcemia in a vaccinee in the Olgin province was excluded because through culture, latex and ELISA methods we were not able to find B antigen.

Broome:
I would like to look a little further into these excluded cases. There were clinically diagnosed two cases of meningococcemia, one fatal in vaccinees and one also fatal in the placebo recipients. They certainly did not have culture confimation, so they did not meet your primary case definition. But in adolescents vaccinated against group C experiencing a group B epidemic, I think those cases are of some consideration as potential group B cases. My expericence with group B antigen testing is that is is not particularly sensitive, certainly not as sensitive as group A or group B antigen detection. I wonder if you could tell us what is the LPS content of the vaccine.

Sierra:
The LPS content of the vaccine is 1% of the total protein amount. If we include the cases without culture the efficacy is around 75%. In one of the three cases mentioned we had not demonstrated B antigens but we have demonstrated other bacterial antigens

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Gotschlich:
You attribute a high importance to the high molecular weight proteins that you add to you vaccine. Could you outline for us the principles of how you obtain these?

Sierra:
We have found one strain with very important growth characteristics in our culture media. We limit in our culture media some of the nutrients, but not iron especially. We are thinking also of doing that in the future. We found that this strain is able to produce higher concentrations of high molecular weight proteins than the other strains. We perform very difficult steps in purification. We purify these high molecular weight proteins, with successive chromatographic steps, and precipitation, and concentration steps. Then, we use them to enrich the preparation of vesicles.

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ORIGINAL ARTICLE

Effect of Eculizumab on Hemolysis and Transfusion Requirements in Patients with Paroxysmal Nocturnal Hemoglobinuria

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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.

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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.

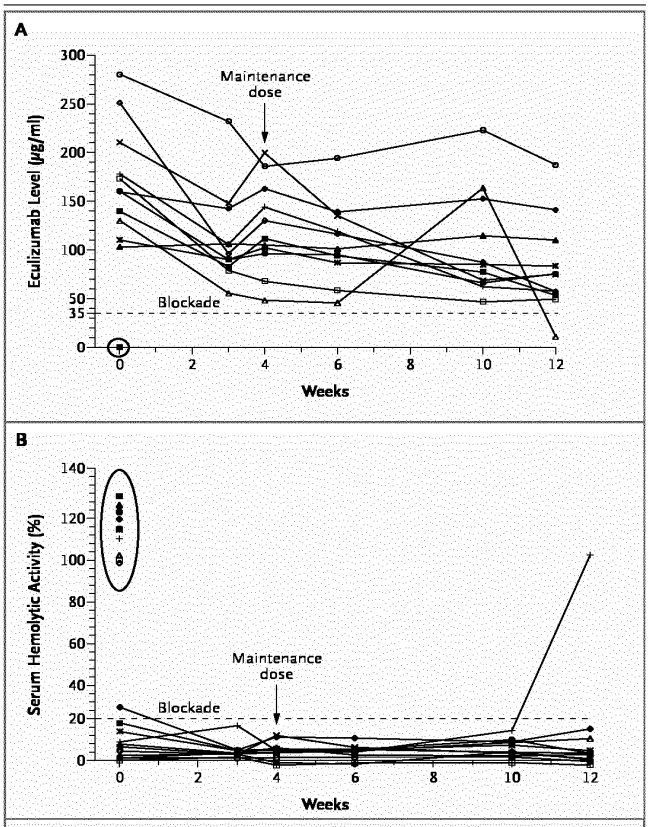


Figure 1. Pharmacokinetic and Pharmacodynamic Analysis of Eculizumab in 11 Patients with Paroxysmal Nocturnal Hemoglobinuria (PNH).

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).

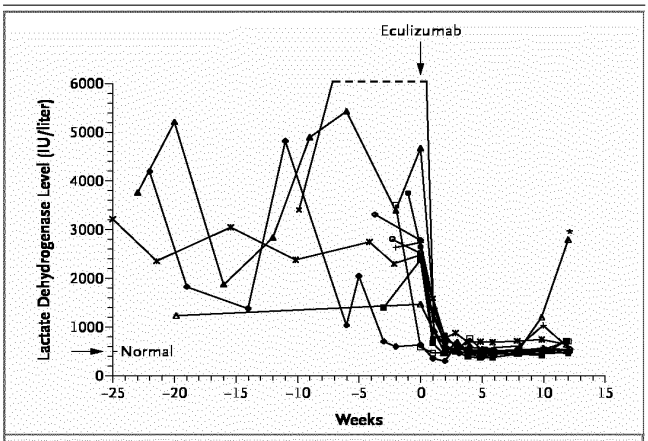


Figure 2. Analysis of Lactate Dehydrogenase Levels, a Biochemical Indicator of Hemolysis, in 11 Patients with Paroxysmal Nocturnal Hemoglobinuria up to 25 Weeks before and during 12 Weeks of Eculizumab Treatment.

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).

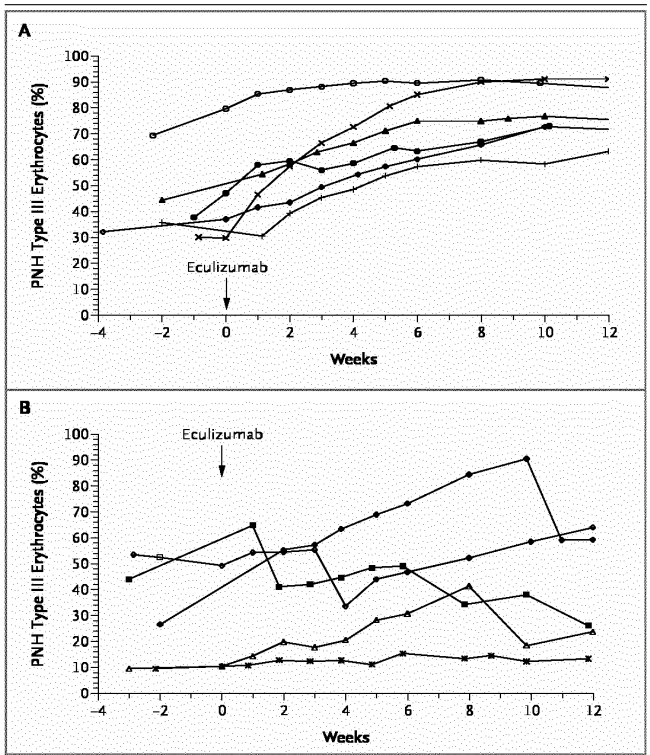


Figure 3. Changes in the Percentage of Paroxysmal Nocturnal Hemoglobinuria (PNH) Type III Erythrocytes during Eculizumab Treatment in Six Patients Who Received No Transfusions during Treatment (Panel A) and Five Patients Who Received at Least One Transfusion after Starting Treatment (Panel B).
The initial 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

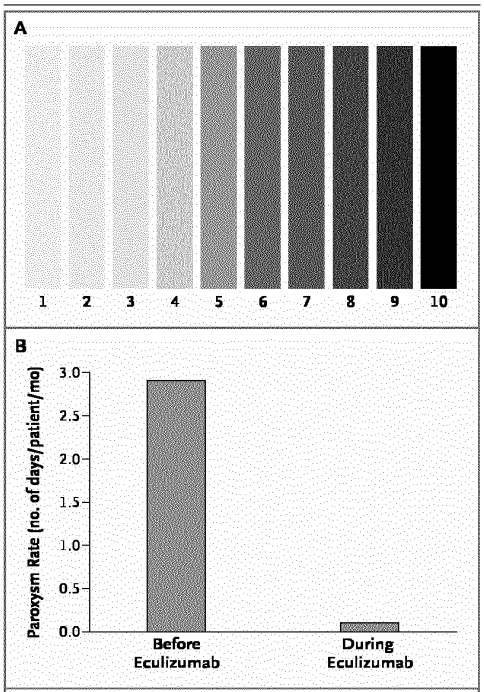


Figure 4. **Incidence of Paroxysms during Eculizumab Treatment.**

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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ADIS R&D PROFILE

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Eculizumab

5G1.1, h5G1.1, Long-Acting Anti-C5 Monoclonal Antibody
5G1-1, Long-Acting Anti-C5 Monoclonal Antibody 5G1.1

Abstract

Eculizumab [*long-acting anti-C5 monoclonal antibody 5G1-1, 5G1.1, h5G1.1, monoclonal antibody 5G1.1, Soliris™*] is a monoclonal antibody that binds to the C5 complement molecule, thereby blocking the progression of the complement cascade at this point. By binding to C5, eculizumab prevents generation of the potent anaphylatoxin C5a and the cytolytic C5b-9 complex, or membrane attack complex. The compound has been filed for approval in the US and the EU for paroxysmal nocturnal haemoglobinuria (PNH), a chronic blood disease caused by a defective enzyme that leads to a lack of protective natural complement inhibitors. It is also being investigated for the treatment of asthma and transplantation.

Eculizumab is a long-acting, humanised version of the anti-C5 antibody [*h5G1.1*]. The short-acting version is called pexelizumab.

In January 2003 Alexion Pharmaceuticals executed a large-scale product supply agreement with Lonza Biologics for the long-term commercial manufacture of eculizumab. Specific terms of the agreement were not disclosed.

Alexion signed an agreement with GTC Biotherapeutics in 2000 for the production of eculizumab in transgenic goats. However, this agreement no longer appears to be active.

Alexion has also filed for approval for eculizumab in PNH in the EU under the centralised procedure. The EMEA has granted Alexion's request for evaluation of the proposed MAA for eculizumab in PNH under the accelerated assessment procedure. This shortens the review part of the MAA procedure.[1,2]

Results from the phase III TRIUMPH trial have been reported; all prespecified primary and secondary endpoints were achieved with statistical significance.[3]

The last patient completed the last visit in the SHEPARD trial in October 2006, and 12-month data is being submitted to regulatory applications. Positive preliminary interim results of 6 months of therapy have been reported. The study has been conducted to collect additional data on eculizumab's safety among haemolytic PNH patients who have undergone transfusion. The trial enrolled 97 patients in the US, Canada, Europe and Australia and includes 12 months of treatment.[4,5]

A pilot, open-label phase Ib trial evaluating eculizumab in 11 patients with PNH has been completed in the UK. All 11 patients in the trial chose to participate in two consecutive 1-year extension studies. One of these is still ongoing, with patients having completed at least 26 months of chronic eculizumab therapy. One- and two-year cumulative results have been presented. The positive findings further supported progression of the clinical development of eculizumab for PNH.[6,7]

Eculizumab has been granted orphan drug status in the US and EU for the treatment of PNH.[8].

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Phase I and II trials in rheumatoid arthritis had previously been completed.

The rationale behind evaluating eculizumab in this indication was that activated terminal complement proteins appear to have a central role in the pathogenesis of immune-mediated joint inflammation, indicating that anti-C5 monoclonal antibodies may have therapeutic potential for the treatment of rheumatoid arthritis.[9]

A phase I pilot trial was completed in patients with dermatomyositis, an inflammatory skin and muscle disorder, for which eculizumab has orphan drug status in the US.

The US FDA granted eculizumab fast-track designation in membranous glomerulonephritis in early 2000 and the drug has orphan drug status in the US for idiopathic membranous glomerular nephropathy.

Both long-acting and short-acting versions of anti-C5 monoclonal antibody 5G1.1 are being developed by Alexion Pharmaceuticals in the US, with the use of technology licensed from Stanford University. Eculizumab is covered by US Patent No. 6 355 245, which extends to C5-specific monoclonal antibodies for the treatment of inflammatory diseases.

The Japanese Patent Office issued patent number 3 734 266 for eculizumab in Japan in February 2006. The patent includes the use of eculizumab to inhibit complement activation in humans, the key mechanism of action used for the treatment of PNH. Corresponding patents have been issued or are pending in Europe, Canada, Australia and other key markets.[10]

1. Profile

1.1 Adverse Events

Eculizumab was safe and well tolerated without any cases of antibodies against the drug detected among 11 patients with paroxysmal nocturnal haemoglobinuria (PNH) in a phase Ib trial. The most common adverse events were headache, upper respiratory infection, sore throat, muscle/joint aches and influenza-like symptoms. Severe adverse events included viral chest infection, dizziness and shivering. Adverse events were similar in type and frequency to those reported with eculizumab or placebo in other controlled trials.[6,11,12] Two-year cumulative results from this study showed that eculizumab continued to be well tolerated. There have been four serious adverse events but no patients have discontinued treatment because of adverse events.[7]

In a multicentre, double-blind, placebo-controlled, ascending-dose trial, 40 patients with rheumatoid arthritis received a single dose of the antibody eculizumab. Within the dose range tested (0.1–8.0 mg/kg), eculizumab was safe and well tolerated. It had no detectable immunogenicity.[13]

Treatment with eculizumab for 2 months was safe and generally well tolerated in a phase I pilot trial in 40 patients with psoriasis. Adverse events included headache and nonspecific pain. 40% of placebo recipients and 10% of the treatment group withdrew from the study before completion.[14]

In a phase I pilot trial, 13 patients with dermatomyositis were randomised to receive eculizumab or placebo. Eculizumab was administered intravenously at a dose of 8 mg/kg once a week for 5 weeks and then once every 2 weeks for up to 2 months. Adverse events were similar in drug-treated and placebo-treated groups. The most common adverse events were rash and headache.[15]

In the double-blind, C99-004 trial, 117 patients with membranous nephritis were randomised to re-

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Table I. Features and properties

CAS number	219685-50-4
WHO ATC code	D05 (Antipsoriatics) L04A (Immunosuppressive Agents) B06 (Other Hematological Agents) G04 (Urologicals) L04A-A (Selective immunosuppressive agents) M01 (Antiinflammatory and Antirheumatic Products) R03 (Drugs for Obstructive Airway Diseases) R07A (Other Respiratory System Products)
EphMRA ATC code	R3 (Anti-Asthma and COPD Products) R7 (Other Respiratory System Products) B6 (All Other Haematological Agents) M1 (Anti-Inflammatory and Anti-Rheumatic Products) G4 (Urologicals) D5 (Nonsteroidal Products for Inflammatory Skin Disorders) L4A (Immunosuppressive Agents)
Originator	Alexion Pharmaceuticals: USA
Highest development phase	Phase Preclinical (USA)
Properties	
Mechanism of action	Complement factor 5A inhibitors
Pharmacodynamics	Prolongs cardiac xenograft survival in an *ex vivo* model; protects red blood cells from activated complement proteins
Route	Unknown route
Adverse events	Occasional: Back pain, Headache, Joint disorders, Muscle pain, Nasopharyngitis, Nausea, Pain, Respiratory tract infections

ceive placebo, eculizumab 8 mg/kg every 4 weeks or eculizumab 8 mg/kg every 2 weeks for 4 months. Eculizumab appeared to be well tolerated, with the most common adverse events being upper respiratory tract infection, diarrhoea and headache.[16]

In the E99-004 trial, 72 placebo and eculizumab patients with membranous nephritis from trial C99-004 were entered into a pre-planned, open-label extension study designed to provide eculizumab 8 mg/kg every 2 weeks for 12 months. The trial is still ongoing and current results show that eculizumab appears to be well tolerated with the most common adverse events being upper respiratory infections, nasal congestion/rhinitis and headache.[16]

In a double-blind, randomised, placebo-controlled phase IIb trial in approximately 350 patients with chronic rheumatoid arthritis undergoing treatment with methotrexate or leflunomide, eculizumab was administered either once per month or biweekly for 6 months. The rates of both serious and common adverse events were similar for placebo and eculizumab. The most common adverse events were upper respiratory tract infections, headache and nausea. Serious adverse events included myocardial infarction, accidental injury and cerebral infarction.[17]

Eculizumab had an adverse event profile comparable to placebo in a trial in 87 patients with paroxysmal nocturnal haemoglobinuria (the TRIUMPH trial). The most frequent adverse events with eculizumab were headache, nasopharyngitis and back pain.[3]

Interim 6-month results from the SHEPHERD trial showed that eculizumab appeared to be safe and well tolerated, with the most frequent adverse events being headache, nasopharyngitis and nausea.[5]

1.2 Pharmacodynamics

1.2.1 Genitourinary Disorders

Mechanism of action: Eculizumab appears to inhibit the production of activated terminal complement proteins, which destroy PNH red blood cells that lack complement protection.[12]

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Table II. Drug development history

Nov 1995	Preclinical development for Rheumatic disorders in the US (Unknown route)
Feb 1996	Preclinical development for Transplant rejection in the US (Unknown route)
Feb 1996	Preclinical development for Adult respiratory distress syndrome in the US (Unknown route)
Aug 1996	Preclinical development for Lupus nephritis in the US (Unknown route)
Jan 1998	IND filed in the US for clinical testing of h5G1.1 in rheumatoid arthritis
May 1998	Preclinical development for Systemic lupus erythematosus in the US (Unknown route)
Aug 1998	Phase-II for Systemic lupus erythematosus in the US (Unknown route)
Aug 1998	Phase-II for Rheumatoid arthritis in the US (Unknown route)
Apr 1999	Alexion has completed a phase I/II trial in rheumatoid arthritis in the US
Jul 1999	No-Development-Reported for Xenotransplant rejection in the US (Unknown route)
Jul 1999	No-Development-Reported for Adult respiratory distress syndrome in the US (Unknown route)
Aug 1999	Phase-I for Lupus nephritis in the US (Unknown route)
Oct 1999	Phase-II for Glomerulonephritis in the US (Unknown route)
Jan 2000	Preclinical development for Psoriasis in the US (Unknown route)
Feb 2000	Preclinical development for Dermatomyositis in the US (Unknown route)
Mar 2000	Eculizumab has received fast-track status for glomerulonephritis in the US
Mar 2000	Phase-II for Lupus nephritis in the US (Unknown route)
Apr 2000	Preclinical development for Bullous pemphigoid in the US (Unknown route)
Aug 2000	Patient enrolment completed in a phase II efficacy trial of h5G1.1 in rheumatoid arthritis
Oct 2000	Phase-I for Psoriasis in the US (Unknown route)
Oct 2000	Phase-I for Dermatomyositis in the US (Unknown route)
Oct 2000	Phase-I for Bullous pemphigoid in the US (Unknown route)
Oct 2000	Eculizumab has received Orphan Drug Status for Dermatomyositis in the US
Feb 2001	Preclinical development for Immune thrombocytopenic purpura in the US (Unknown route)
Mar 2001	Eculizumab has received orphan drug status for idiopathic membranous glomerular nephropathy in the US
Jan 2002	A phase IIb trial of h5G1.1 has commenced in rheumatoid arthritis
Jan 2002	Alexion has completed a pilot phase I trial in Dermatomyositis in the US
Jan 2002	Phase-II/III in Rheumatoid arthritis in the US (unspecified route)
Jan 2002	Phase-II/III in Rheumatoid arthritis in Canada (unspecified route)
Mar 2002	Enrolment has been completed in a phase II trial for membranous nephritis
Jan 2003	Alexion Pharmaceuticals has entered into a large-scale product supply agreement with Lonza Biologics
Jan 2003	Alexion has completed enrolment in a phase IIb trial for rheumatoid arthritis in the US and Canada
Jul 2003	Suspended – Phase-I for Dermatomyositis in the US (unspecified route)
Aug 2003	Eculizumab has received orphan drug status for paroxysmal nocturnal haemoglobinuria in the US
Dec 2003	Eculizumab has received orphan drug status for Paroxysmal nocturnal haemoglobinuria in the EU

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Table II. Contd

Jul 2004	The US FDA has approved two protocols, under a Special Protocol Assessment (SPA), for the pivotal phase III programme of eculizumab in Paroxysmal Nocturnal Haemoglobinuria
Nov 2004	Alexion has initiated enrolment in the TRIUMPH trial for paroxysmal nocturnal haemoglobinuria in Australia, Canada, Europe and the US
Nov 2004	Phase-III in Paroxysmal nocturnal haemoglobinuria in Canada (IV-infusion)
Nov 2004	Phase-III in Paroxysmal nocturnal haemoglobinuria in European Union (IV-infusion)
Nov 2004	Phase-III in Paroxysmal nocturnal haemoglobinuria in the US (IV-infusion)
Nov 2004	Phase-III in Paroxysmal nocturnal haemoglobinuria in Australia (IV-infusion)
Jan 2005	Alexion has initiated enrolment in the phase III SHEPHERD trial for paroxysmal nocturnal haemoglobinuria in Australia, Canada, Europe, and the US
Apr 2005	Alexion Pharmaceuticals has completed enrolment in the TRIUMPH trial for Paroxysmal Nocturnal Haemoglobinuria in the US, Canada, Europe and Australia
Jul 2005	Alexion has completed randomisation in the phase III TRIUMPH trial for paroxysmal nocturnal haemoglobinuria in Australia, Canada, Europe and the US
Sep 2005	Alexion Pharmaceuticals has completed enrolment in the SHEPHERD trial for Paroxysmal Nocturnal Haemoglobinuria in the US, Canada, Europe and Australia
Sep 2005	Discontinued – Phase-II for Lupus nephritis in the US (unspecified route)
Sep 2005	No development reported - Preclinical for Immune thrombocytopenic purpura in the US (unspecified route)
Sep 2005	Discontinued – Phase-II for Systemic lupus erythematosus in the US (unspecified route)
Sep 2005	Discontinued – Phase-II/III for Rheumatoid arthritis in the US (IV-infusion)
Sep 2005	Discontinued – Phase-I for Psoriasis in the US (unspecified route)
Sep 2005	Discontinued – Phase-I for Dermatomyositis in the US (unspecified route)
Sep 2005	Discontinued – Phase-I for Bullous pemphigoid in the US (unspecified route)
Sep 2005	Discontinued – Phase-II/III for Rheumatoid arthritis in Canada (IV-infusion)
Sep 2005	Discontinued – Phase-II for Glomerulonephritis in the US (unspecified route)
Mar 2006	This compound is still in active development for transplant rejection
Mar 2006	Prein Asthma in the US (unspecified route)
Aug 2006	Eculizumab has received accelerated assessment status for paroxysmal nocturnal haemoglobinuria in Europe
Sep 2006	Preregistration for Paroxysmal nocturnal haemoglobinuria in the US (IV-infusion)
Sep 2006	Preregistration for Paroxysmal nocturnal haemoglobinuria in European Union (IV-infusion)
Nov 2006	Alexion has been granted a priority review for eculizumab by the FDA
Nov 2006	Discontinued – Preclinical for Adult respiratory distress syndrome in the US (unspecified route)
Nov 2006	Prein Transplant rejection in the US (unspecified route)
Nov 2006	Discontinued – Preclinical for Immune thrombocytopenic purpura in the US (unspecified route)

In C5-deficient mice reconstituted with human C5, eculizumab potently inhibited the complement system when administered IV or SC at equivalent doses.[18]

1.2.2 Immunological Disorders

In C5-deficient mice reconstituted with human C5, eculizumab potently inhibited the complement system when administered IV or SC at equivalent doses.[18]

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1.2.3 Respiratory Tract Disorders

In C5-deficient mice reconstituted with human C5, eculizumab potently inhibited the complement system when administered IV or SC at equivalent doses.[18]

1.2.4 Rheumatic Disease

In C5-deficient mice reconstituted with human C5, eculizumab potently inhibited the complement system when administered IV or SC at equivalent doses.[18]

1.2.5 Transplant Rejection

In C5-deficient mice reconstituted with human C5, eculizumab potently inhibited the complement system when administered IV or SC at equivalent doses.[18] Eculizumab blocked human complement-mediated haemolytic activity and C5a generation *in vitro*.

In an *ex vivo* model of pig-to-human xenotransplantation, the addition of eculizumab (50–200 μg/mL) to human blood prior to perfusion dose-dependently prolonged graft survival time compared with no treatment. No signs of hyperacute rejection were observed in the two hearts that were perfused with blood containing the highest dose of antibody. Eculizumab reduced total complement activity, and the two higher doses reduced fluid phase C5b-9 levels compared with no treatment, indicating that the prevention of C5b-9 production correlates with increased heart survival.[19]

1.3 Therapeutic Trials

1.3.1 Genitourinary Disorders

In the double-blind, C99-004 trial, 117 patients with membranous nephritis were randomised to receive placebo, eculizumab 8 mg/kg every 4 weeks or eculizumab 8 mg/kg every 2 weeks for 4 months. The primary efficacy endpoint, 24-hour urinary protein, was not significantly different between study groups. However, subset analyses showed that patients who appeared to benefit most clinically also had more complete complement inhibition; an important finding as to the pathology of the disease and to the effectiveness of eculizumab treatment.[16]

In the E99-004 trial, 72 placebo and eculizumab patients with membranous nephritis from trial C99-004 were entered into a pre-planned, open-label extension study designed to provide eculizumab 8 mg/kg every 2 weeks for 12 months. The trial is still ongoing and after 9 months of therapy, eculizumab administration was associated with significant improvements in proteinuria, urinary protein/creatinine ratio and serum albumin. At the end of 12 months of therapy, urinary protein decreased 42% ($p < 0.001$), urinary protein/creatinine ratio decreased 45% ($p < 0.001$), serum cholesterol decreased 10% ($p = 0.005$), serum triglycerides decreased 16% ($p = 0.008$) and serum albumin decreased 14% ($p < 0.001$) in the 39 patients for whom 1 year of follow-up data was available. Remission rate was examined prospectively using the widely employed clinical definition of remission in patients with nephrotic membranous nephritis that requires both a 50% reduction in proteinuria and a 24-hour proteinuria <3.5g. For the 55 evaluable E99-004 patients who were treated with eculizumab for 6 months or longer, eculizumab treatment was associated with a 27% 12-month remission rate by Kaplan-Meier analysis.[16]

1.3.2 Haematological Disorders

Cumulative 1-year results from an ongoing open-label, phase Ib trial showed that eculizumab produced a rapid clinical response within a week of administration that lasted over the course of at least 1 year. Eculizumab significantly reduced the need for blood transfusions, intravascular haemolysis and haemoglobinuria among 11 patients with PNH. This was accompanied by an associated improvement in the quality of life, which was maintained for at least 1 year of continuous treatment. All patients received four once-weekly intravenous (IV) infusions of eculizumab 600mg, followed by a 900mg dose 1 week later and then by 900mg every other week through week 12. Levels of lactate dehydrogenase were significantly decreased from a mean of 3111 IU/L at baseline to 594 IU/L during treatment. The percentage of PNH type III erythrocytes increased significantly from a mean of 36.7% at baseline to 59.2% at the end of 12 weeks of treatment. In addition, the

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mean and median transfusion rates were reduced from baseline values of 2.1 and 1.8 units per month, respectively, to 0.6 and 0.0 units per month, respectively, during eculizumab therapy. In nine patients for whom urine scores were assessed, the mean paroxysm rate (defined as the mean number of days in paroxysm per patient per month) was significantly reduced from 2.9 days to 0.12 day. During treatment, there were significant improvements in measurements of the quality of life.[6,11,12,20] Two-year cumulative results from this study showed that eculizumab was associated with statistically significant reductions in haemolysis and in the need for blood transfusions. Two-year results showed the percentage of PNH type III erythrocytes to have increased significantly to 58.9%. The mean and median transfusion rates were reduced to 0.4 and 0.3 units/patient/month, respectively, through the 23- to 26-month treatment period.[7]

Eculizumab achieved all pre-specified primary and secondary endpoints in the TRIUMPH trial with statistical significance. The median transfusion rate for placebo was 10 units/patient compared with 0 units/patient for eculizumab. No patients in the placebo group achieved haemoglobin stabilisation, compared with 49% of patients in the eculizumab group. The TRIUMPH trial enrolled 87 patients with paroxysmal nocturnal haemoglobinuria.[3]

All pre-specified primary and secondary efficacy endpoints were achieved with statistical significance in the SHEPHERD trial at 6 months; lactate dehydrogenase was reduced by 87%.[5]

1.3.3 Immunological Disorders

A single dose of eculizumab up to 8 mg/kg was well tolerated by 24 lupus patients in a phase I double-blind, placebo-controlled ascending dose trial. A single 8 mg/kg dose of eculizumab was associated with a significant reduction in the incidence of proteinurea, an objective measurement of kidney dysfunction. A single dose of eculizumab potently blocked complement activity in patients for up to 2 weeks.[21]

1.3.4 Rheumatic Disease

In a multicentre, double-blind, placebo-controlled, ascending-dose trial, 40 patients with rheumatoid arthritis received a single dose of the antibody eculizumab (0.1–8.0 mg/kg). Eculizumab dose-dependently blocked complement activity for ≤2 weeks. The highest dose (8.0 mg/kg) significantly reduced C-reactive protein levels (the most objective component of the American College of Rheumatology's disease parameters), whereas in placebo recipients, C-reactive protein increased 24%. Eculizumab also reduced some of the more subjective measures of disease activity, such as tender joint count, swollen joint count, patient's global assessment of disease and patient's global assessment of pain.[13]

In a multicentre study, 209 patients with mild-to-moderate rheumatoid arthritis were randomised to receive IV eculizumab 8 mg/kg once weekly for 4 weeks then once monthly (induction/monthly group), 8 mg/kg once weekly for 4 weeks then once every 2 weeks (induction/biweekly group) or 8 mg/kg once every 2 weeks (biweekly group), or placebo. After 3 months' treatment, a higher proportion of patients in the induction/monthly, compared with the placebo, group met American College of Rheumatology 20% improvement criteria (ACR 20) [43% vs 20%]. Compared with the placebo group, patients in the induction/monthly and induction/biweekly groups had greater reductions from baseline in C-reactive protein levels.[22]

In a double-blind, randomised, placebo-controlled phase IIb trial in 368 patients with chronic rheumatoid arthritis undergoing treatment with methotrexate or leflunomide, eculizumab was administered either once per month or biweekly for 6 months. Monthly administration caused a significant, moderate improvement in ACR20 (34%), compared with placebo (22%), at 6 months. In addition, erythrocyte sedimentation rate (ESR) was significantly reduced. Biweekly administration of eculizumab did not significantly change ACR20 compared with placebo.[17,23]

1.3.5 Skin Disorders

Psoriasis: Treatment for 2 months with eculizumab did not significantly improve Psoriasis Area and Severity Index (PASI) scores in a study in 40 patients with severe psoriasis. However, trends

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towards improvement were observed in certain measures of disease activity. Haemolytic activity and deposition of activated terminal complement in psoriatic plaques were reduced in a dose-dependent manner.[14]

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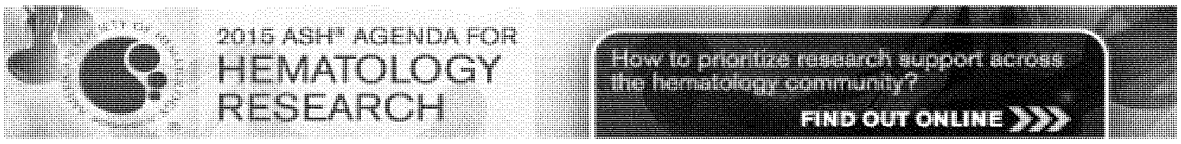
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Sustained Control of Hemolysis and Symptoms and Reduced Transfusion Requirements over a Period of 2 Years in Paroxysmal Nocturnal Hemoglobinuria (PNH) with Eculizumab Therapy.

Anita Hill, Dupe Elebute, Judith C. Marsh, Stephen J. Richards, Bruce McNutt, Beth E. Petro, Russell P. Rother and Peter Hillmen

Blood 2004 104:2823;

Abstract

Paroxysmal nocturnal hemoglobinuria (PNH) is characterized by intravascular hemolysis and venous thrombosis. Deficiency of the terminal complement inhibitor CD59 from PNH red cells results in complement-mediated hemolysis. Eculizumab, a humanized monoclonal antibody that inhibits terminal complement by binding to C5, is effective at controlling intravascular hemolysis in PNH. We now report that 10 of the 11 patients from an initial 3 month study have continued to receive 900mg eculizumab every other week for 2 years. The remaining patient stopped eculizumab after 23 months despite effective control of intravascular hemolysis, as the patient continued to be transfused even after erythropoietin therapy. This patient had the most severe hypoplasia at the start of eculizumab therapy with a platelet count below $30x10^9/l$, suggesting that the ongoing transfusions were due to the bone marrow failure and not continuing hemolysis. Eculizumab was safe and well tolerated with two reported SAEs in the last year, neither of which were attributed to the drug. The dramatic improvement in various parameters of hemolysis persisted during the 2 year treatment period for all patients. Mean LDH levels decreased from 3111 +/− 598 U/L over the 12 months prior to treatment to 634 +/− 34 U/L up to 24 months following treatment (p=0.002). PNH red cells with a complete deficiency of GPI-linked proteins (Type III red cells) progressively increased during the treatment period from a mean of 36.7% to 58.9%

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($p=0.001$) while partially deficient PNH red cells (Type II) increased from 5.3% to 8.7% ($p=0.01$). There has been no change in the proportion of PNH neutrophils in any of the patients during eculizumab therapy indicating that the increase in the proportion of PNH red cells is due to a reduction in hemolysis and transfusions rather than a change in the PNH clone(s) itself. The mean and median transfusion rates decreased from 2.1 and 1.8 units/patient/month to 0.4 and 0.3 units/patient/month respectively ($p<0.001$). Three patients remained transfusion independent during the whole 2 year treatment period and the other 7 patients remaining on eculizumab all had at least a three-fold reduction in transfusion requirements. No patients have had a thrombosis during the study period, including 5 patients who were not on warfarin therapy. Prior to eculizumab, some patients suffered with abdominal pain, dysphagia and erectile dysfunction. Following the first dose of eculizumab there has been complete and sustained resolution of these symptoms. In addition, other symptoms related to hemolysis, such as lethargy, have shown a sustained marked improvement. These classical PNH symptoms have been attributed, at lease in part, to the release of free hemoglobin (Hb) during intravascular hemolysis which overwhelms the protective Hb scavenging mechanisms. Free Hb efficiently absorbs and depletes nitric oxide (NO) which is a critical regulator of smooth muscle contraction, vascular tone and platelet activation. The potential role of NO in the symptomologies of PNH are currently under investigation. In summary, we report that eculizumab is well tolerated and has persisting efficacy as demonstrated by decreased hemolysis and transfusions, as well as improvement in the symptoms of PNH, for over 2 years of therapy.

2005, The American Society of Hematology

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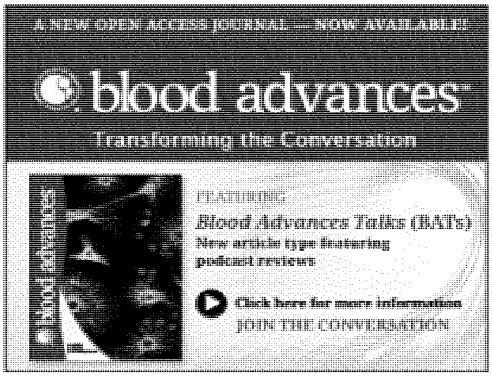
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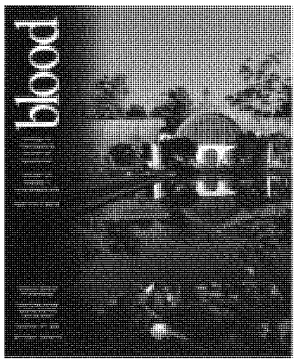


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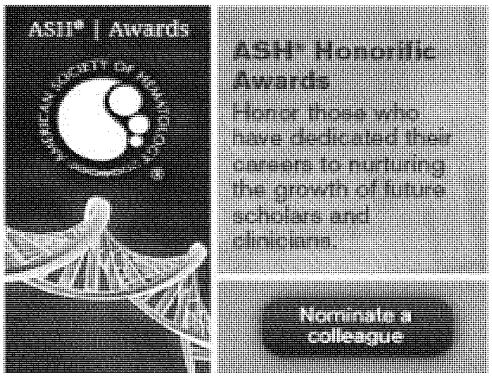
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Case report

Complement C7 deficiency presenting as recurrent aseptic meningitis

Michael Corvini, MD; Christopher Randolph, MD; and Steven I. Aronin, MD

Background: Complement deficiency states are rare inherited disorders that may predispose affected individuals to angioedema, collagen vascular disease, or infection due to encapsulated organisms, especially *Neisseria meningitidis*.

Objectives: To report the case of a 36-year-old man of Irish descent with recurrent culture-negative neutrophilic meningitis, to offer potential reasons for the inability to recover a causative pathogen, and to review the genetics and prevalence of complement deficiency states, the methods of screening for such deficiencies, the features of meningococcal infection as they relate to such deficiencies, and management strategies for clinicians caring for patients with such deficiencies.

Methods: The patient presented in 1988 and again in 2002 with culture-negative neutrophilic meningitis. His second episode was characterized by a rash suggestive of meningococcal infection, prompting immunologic evaluation.

Results: Immunologic evaluation revealed an undetectable CH_{50} level. Levels of C1, C2, and C5 through C9 were normal except for C7, which was undetectable. Further testing revealed that the patient's sister was also C7 deficient.

Conclusions: Complement component deficiencies are relatively rare; individuals with collagen vascular disease and systemic neisserial infection should be screened using either the CH_{50} or the APH-50 assay. Key to the management of a late–complement component–deficient host is counseling, education about meningococcal infection, and discussions about the potential benefits of chemoprophylaxis and immunoprophylaxis. The ability to detect the bacterial cause of meningitis in such patients is organism dependent and may be influenced by factors such as cerebrospinal fluid bacterial concentration and previous antibiotic drug exposure.

Ann Allergy Asthma Immunol. 2004;93:200–205.

INTRODUCTION

The complement pathways play an integral role in the immune system.[1,2] Deficiencies in any complement components or pathologic activation of a normally functioning complement system can yield clinical disease, typically manifested as angioedema, collagen vascular disease, or infection. The seventh component of complement, C7, is part of the membrane attack complex (MAC) required for complement-dependent bactericidal activity, including defense against neisserial infection.[3,4] In this article, we describe a patient with C7 deficiency presenting with inflammatory arthritis and recurrent culture-negative neutrophilic meningitis.

CASE REPORT

A 36-year-old man was well until April 2002, when he developed transient pharyngitis followed by fever (temperature, 102.2° F), chills, sweats, and malaise. Subsequently, he developed headache, light sensitivity, neck pain and stiffness, nausea, vomiting, and right hip pain with an overlying rash. Five days into his illness, he presented to the emergency department (ED) with ongoing symptoms and a rash now involving his left shoulder, chest, and abdomen.

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The patient was hospitalized 14 years earlier for an illness consistent with bacterial meningitis by clinical presentation and lumbar puncture (LP) but with negative cerebrospinal fluid (CSF) and blood culture findings. He takes no medications, including nonsteroidal anti-inflammatory agents, and has no history of craniofacial trauma or drug allergies. His father died at the age of 45 years of a myocardial infarction. There is no family history of immunodeficiency, meningitis, arthritis, or other inflammatory or inherited disease. He lives with his wife and daughter in Connecticut, smokes cigarettes, and drinks alcohol, but he has never used illicit drugs. He is monogamous with his wife and has no history of sexually transmitted diseases. He is a plumber, has not traveled outside New England, and has no history of tick exposure. He did have contact with rodent droppings 1 day before becoming febrile.

In the ED, the patient appeared ill. His temperature was 101.0° F and his heart rate was 110/min. His blood pressure was normal without orthostatic changes, and his respiratory rate and oxygen saturation were normal. Nuchal rigidity and photophobia were present. Neurologic examination revealed only a positive Brudzinski sign. He had dry mucous membranes, flat neck veins, and no lymphadenopathy. His lungs were clear, his heart was tachycardic, and the results of abdominal examination were normal. There was markedly limited range of motion of the right hip and left shoulder. The

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affected joints were warm but without appreciable erythema or effusion. A scattered, tender, purpuric-petechial rash was distributed over the chest, left shoulder, arms, abdomen, right hip, and groin. The palms and soles were spared.

The leukocyte count was 18,000/mL, with 62% segmented forms and 30% bands; the hematocrit level was 43.4%; andthe platelet count was 228,000/mL. Findings from a comprehensive metabolic profile, coagulation indices, and urinalysis were unremarkable. Findings from chest radiography were normal, and electrocardiography showed sinus tachycardia. Lumbar puncture disclosed hazy CSF, with an opening pressure of 123 mm H_2O, a protein level of 186 mg/dL (normal, 14–45 mg/dL), a glucose level of 49 mg/dL (normal, 45–75 mg/dL), a red blood cell (RBC) count of 128/mL, a white blood cell (WBC) count of 491/mL (90% neutrophils), and moderate polymorphonuclear cells but no organisms on Gram stain.

One hour after ED arrival, a blood sample was cultured, and then 1 g of ceftriaxone was administered. One hour later, LP was performed, and then 1 g of vancomycin and an additional 1 g of ceftriaxone were administered, and a second blood sample was cultured. The patient was placed on "droplet precautions" in the intensive care unit, vigorously hydrated, and empirically treated for bacterial meningitis with ceftriaxone and vancomycin. His meningitic symptoms resolved and his vital signs normalized within 12 hours, whereas his arthritic symptoms recovered more slowly. Left shoulder aspirate yielded 4 mL of frank pus, a WBC count of 234,133/mL (90% neutrophils), and an RBC count of 800/mL. Right hip aspirate was serosanguinous, with a WBC count of 25/mL (45% neutrophils) and an RBC count of 24,747/mL. No crystals or organisms were identified from either specimen. Skin lesion Gram staining was negative for organisms. One of 2 bottles from the first blood culture grew coagulase-negative staphylococcus. Both bottles from the second blood culture were negative. Cerebrospinal fluid, skin lesion, and synovial fluid cultures were negative. Serologic test results for Lyme disease, Rocky Mountain spotted fever, and leptospirosis were negative. Findings from CSF latex agglutination for *Streptococcus pneumoniae, Neisseria meningitidis,* and *Haemophilus influenzae* were negative. The CH_{50} level, determined by enzyme colorimetry, was undetectable at less than 10 U/mL (normal, 26–58 U/mL; Quest Diagnostics Reference Laboratory, Wallingford, CT). Levels of C1, C2, and C5 through C9 (protein quantitation by radial immunodiffusion) were normal except for C7, which was undetectable at less than 2 mg/dL (normal, 4–11 mg/dL; Quest Diagnostics Reference Laboratory). C1 esterase inhibitor, antinuclear antibody, antibody to double-stranded DNA, rheumatoid factor, quantitative immunoglobulin, and human immunodeficiency virus antibody levels were all normal.

The patient completed 14 days of parenteral antibiotic drug therapy and was given the tetravalent meningococcal vaccine and a medical identification bracelet (MedicAlert; MedicAlert Foundation International, Turlock, CA) before hospital discharge. CH_{50} and C7 levels measured during his convalescence remained undetectable. Family members were screened for complement deficiencies (Table 1).

DISCUSSION

Genetically determined complement deficiencies have been described for nearly all of the complement components, including C7, which is inherited in an autosomal-recessive manner.[3] The C6 and C7 genes are polymorphic, closely linked on 5p13, and are close homologues of the C8α, C8β, and C9 genes.[5] The molecular bases of subtotal and total C6 and C7 deficiency have been reported previously.[6,7] Numerous polymorphic DNA markers have been reported for the C6 and C7 genetic region, illustrating the genetic heterogeneity associated with defects in these complement components.[8–15] Such polymorphisms have been described in individuals of Japanese, South African, Russian, Moroccan Sephardic Jewish, and Irish ancestry. The patient's parents emigrated from Ireland, and both must have been carriers for C7 deficiency. His father is deceased, but his mother's serum C7 level was compatible with a carrier state. Further testing revealed that his sister is also C7 deficient (Table 1).

Complement deficiency states are typically identified via the CH_{50} or the APH-50 assay. All of the terminal components of complement (C5-C9) are required to yield normal CH_{50} and APH-50 levels; thus, both assays provide a sensitive means of screening for late–complement component–deficient (LCCD) hosts.[4] Using these assays, the prevalence of complement deficiency states in the general population has been estimated to be 0.03%.[16] Given the rarity of complement

Table 1. Results of Complement Studies Performed on the Proband and His Family Members

Source	Age, y	Health status	Complement, mg/dL								
			CH_{50}*	C2	C3	C4	C5	C6	C7	C8	C9
Proband	36	Normal	<10	3.4	211	25	22	10.6	<2	8.6	73
Daughter	5	Normal	53	2.1	113	17	10	6	6	5.5	24
Brother	39	Diabetes	60	3	117	16	14	6.6	4	5.6	29
Sister	38	Normal	<10						<2	8.7	44
Mother	61	Normal	48	2.5			15	6	2	4.6	29
Paternal aunt	62	Normal	51	2.8			17	3.6	4	5	25
Normal range			26–58	1.6–3.5	88–201	16–47	6–20	3.6–7.5	4–11	2.7–6	6–29

* Measured in units per milliliter.

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deficiency states, the utility of screening assays is greatest in disease populations known to contain the clinical correlates of complement protein abnormalities (patients with collagen vascular disorders and those with systemic neisserial infection).[17] For patients with meningococcal disease, the prevalence of an underlying complement deficiency state varies worldwide (10%–15% in the United States) and is inversely related to the incidence of meningococcal disease by country.[3,17] Such relationships must be considered when making recommendations regarding the value of screening an individual patient with meningococcal infection for an underlying complement deficiency. A CH_{50} level was obtained on our patient because of his history of recurrent meningitis and a rash that was most consistent with meningococcal disease. Once the CH_{50} level indicated a complement deficiency, assays for the individual complement components were performed, and a C7 deficiency was identified. The patient's family was also screened using the CH_{50} assay and then the targeted C7 assay (Table 1).

Neisseria meningitidis causes approximately 80% of the infections in patients with any complement deficiency, and the remainder are due to other encapsulated organisms, such as *S pneumoniae* and *H influenzae*.[17] Infections in LCCD hosts, though, are nearly exclusively due to *N meningitidis*. This can be explained by understanding that the terminal complement components compose the MAC, which is responsible for complement-mediated bacterial cell lysis. Patients who are LCCD, therefore, are unable to assemble the MAC and are unable to effect complement-mediated cell lysis. *Neisseria meningitidis* (in contrast to all gram-positive and some gram-negative organisms) is extremely sensitive to complement-mediated killing,[3] and, thus, LCCD patients are particularly susceptible to meningococcal infection.

The frequency of meningococcal disease is 10,000-fold greater in LCCD hosts vs healthy hosts (57% vs 0.0072%).[17] Furthermore, in LCCD hosts there is a later median age of first meningococcal infection (17 vs 3 years), a higher relapse rate (7.6% vs 0.6%), a higher recurrence rate (41% vs 0.34%), and a 10-fold lower mortality rate (1.5% vs 19%) compared with healthy hosts.[17] Ross and Densen[3] explain these differences using hypothetical relationships among serum bactericidal activity, protective antibody, meningococcal exposure, and age. According to this model, both LCCD and healthy newborns express high levels of maternal protective antibody and serum bactericidal activity. Thereafter, both of these levels fall to their nadir such that, in healthy hosts, there is a narrow window of susceptibility for meningococcal infection between 6 months and 2 years of age. During this window, asymptomatic exposure to *N meningitidis* begins to occur, yielding a rise in serum bactericidal activity and protective antibody. In the LCCD host, serum bactericidal activity remains undetectable, yielding a lifelong, broad window of susceptibility to meningococcal infection. This lifelong window of susceptibility explains why LCCD patients are at increased risk of meningococcal infection, have an older median age of first infection, and have higher recurrence rates compared with healthy hosts.

In terms of the lower mortality rate noted in LCCD patients, some researchers[18] attribute this to ascertainment bias (complement deficiency is usually diagnosed in survivors of meningococcal disease, and complement testing is rarely performed in patients with fatal disease). Those who believe that this difference in prognosis is real offer 2 possible explanations. First, the lack of a MAC, as seen in LCCD hosts, results in preservation of the bacterial cell membrane, and, thus, there is no endotoxin release, septic shock, or death.[18] Second, in contrast to the sporadic cases in healthy hosts, episodes of meningococcal infection in LCCD patients occur in the presence of protective antibody, thus allowing opsonization to occur, which may clear some of the organism by phagocytosis.[3] Assuming that our patient had meningococcal infection at the age of 22 years, and again now, his older age at the first episode, recurrent disease, and survival are all typical features of meningococcal infection in an LCCD host.

Presumed and confirmed meningococcal infection in C7-deficient hosts has been previously reported,[3–8,18–33] mostly in the form of case reports containing incomplete clinical details. Review of 24 C7-deficient patients with a first episode of presumed or documented meningococcal infection reveals no sex preference and a median age of 15 years (range, 3–40 years).[5,7,8,18,22–33] Patients' ethnic origins include Irish, Moroccan, Russian, Japanese, Arab, and Yugoslav. Twenty (83%) of the 24 patients had clinical or laboratory evidence of meningitis either alone or in conjunction with arthritis (n = 4; 17%) or myopericarditis (n = 2; 8%). Three patients had chronic meningococcemia without central nervous system involvement. Rash was reported in 12 patients, and it was characterized as petechial or purpuric in 11 of the 12 patients. The CSF formula was not provided for these 24 patients, but up to 20% of cases of meningococcal meningitis from the literature have a CSF WBC count less than 1,000/mL, and 24% to 55% have a normal CSF glucose concentration.[34–37] Of the 24 C7-deficient patients we reviewed, the clinical diagnosis of meningococcal infection was confirmed by bacterial culture in 15 (63%). Even lower rates of microbiologic confirmation (20%–40%) were reported for 77 episodes of meningococcal infection in 30 patients with either C7 or C8 deficiency[18]; the authors attribute this to exposure to high-dose penicillin 1 to 2 hours before blood and CSF cultures were obtained. Finally, 12 of the 24 C7-deficient patients we reviewed had recurrent meningococcal infection, 3 had multiple recurrences, and all 24 responded to parenteral antibiotic drug therapy without clinical sequelae. Therefore, our patient's age, ethnicity, clinical presentation (now and 14 years ago), CSF formula, and response to therapy are all compatible with recurrent meningococcal infection in a C7-deficient host.

Although it is suggestive that our patient had meningococcal infection, no such organism was identified using routine methods. The possibility remains that he was infected by a bacterium other than *N meningitidis* or by a nonbacterial pathogen or that he experienced a noninfectious meningitis syndrome. Tick-borne bacterial infections, such as Lyme disease, Rocky Mountain spotted fever, and ehrlichiosis, typ-

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ically occur in the late spring and summer months and can manifest as aseptic lymphocytic meningitis with or without joint involvement. Our patient exhibited several features that did not support the diagnosis of a tick-borne infection: his illness occurred in April, he had no history of a tick bite, his rash differed from the characteristic rash of Lyme disease and Rocky Mountain spotted fever, his arthritis developed during the acute phase of illness (as opposed to Lyme arthritis, which is a late manifestation), and he had neutrophilic CSF pleocytosis. Results of short-term serologic testing for the agent of Lyme disease and Rocky Mountain spotted fever were negative. The CSF and Lyme antibody and Lyme polymerase chain reaction on the synovial fluid, however, was not performed. Aseptic meningitis can also be a manifestation of leptospirosis and lymphocytic choriomeningitis. Such infections typically occur during the second phase of disease, 1 to 2 weeks after exposure to rodents or rodent droppings. Our patient's exposure to rodent droppings occurred only 1 day before he became ill, thus making these infections very unlikely. Noninfectious processes that have been associated with aseptic meningitis include collagen vascular disorders, medications,[38] adult-onset Still disease,[39] and complement deficiency itself.[40] In our patient, serologic test results for collagen vascular disorders were negative, he was not taking any medications before becoming ill, and the appearance of his rash was not characteristic of adult-onset Still disease. Furthermore, meningitis as the initial manifestation of adult-onset Still disease is exceedingly rare, and, nearly 2 years later, our patient has not had any further evidence of arthritis.

Perhaps a bacterial etiology could not be identified because the organism was not present at the time of sampling or because the diagnostic tests used could not detect its presence. For pediatric patients with proven bacterial meningitis, blood culture detects the causative organism in 40% to 90%, CSF Gram stain in 60% to 90%, CSF culture in 70% to 85%, and CSF antigen testing in 50% to 100% of cases.[41] Polymerase chain reaction testing for specific meningeal pathogens[42–44] and for a broad range of bacterial pathogens[45] has also been used to determine the bacterial etiology of cases of meningitis. Several investigators[42–44] have shown that compared with CSF culture, polymerase chain reaction is a highly sensitive and specific means of diagnosing meningococcal meningitis. As more experience is gained with this diagnostic test, it will undoubtedly become a powerful tool in the diagnostic approach to patients with meningitis syndromes.

Although the CSF Gram stain is positive in 60% to 90% of cases of bacterial meningitis, results vary by organism and by the concentration of bacteria in the CSF. The CSF Gram stain is positive by organism as follows: *S pneumoniae,* 90%; *H influenzae,* 86%; *N meningitidis,* 75%; gram-negative bacilli, 50%; and *Listeria monocytogenes,* less than 50%.[46] Bacterial concentrations of 10^5 CFU/mL or greater are associated with positive CSF Gram staining in up to 97% of cases, whereas concentrations of 10^3 CFU/mL or less are associated with a positive Gram stain result in approximately 25% of cases.[47] There are limited data defining the CSF bacterial concentration in cases of meningitis due to *N meningitidis*. One study[48] of 9 patients with meningococcal meningitis found a mean concentration of 1.27×10^5 CFU/mL. In this small study, as few as 1,500/mL of meningococcal colonies were found (quantities clearly below the threshold of detection by Gram staining). Low CSF bacterial concentrations could explain why the Gram stain was negative in our patient, but this would not necessarily explain the negative culture findings.

The last potential reason why no bacterial cause was identified relates to the impact of antibiotic drug therapy on the results of diagnostic tests. Although the receipt of antibiotics before CSF sampling does not significantly alter the CSF WBC count, glucose level, or protein concentration,[49] such therapy can certainly affect blood culture and CSF Gram stain and culture results. In the presence of antibiotics, the ability to detect an organism by CSF Gram stain and culture drops to 40% to 60% and less than 50%, respectively,[50] and the rate of recovery from blood culture drops to less than 5%.[51] How fast antibiotics negate the ability to make a microbiologic diagnosis by CSF analysis is not known. A study of experimental pneumococcal meningitis in rabbits illustrates a 2-log drop in CSF bacterial concentration every hour after penicillin administration such that one would expect to see negative smears by 4 hours and sterilization by 8 hours.[52] This study is often quoted as evidence for a 4- to 8-hour window between antibiotic administration and LP before it is too late to make a microbiologic diagnosis. However, it is unclear whether these data can be extrapolated to infection due to organisms other than *S pneumoniae* or to humans.

In humans, the use of repeated LP illustrates that 90% to 100% of CSF cultures are sterile by 24 to 36 hours after the administration of appropriate antibiotics.[50] More precise human data on the rate of CSF sterilization have not been established, but in a recent retrospective study[53] of 128 cases of pediatric meningitis, the authors found that the interval between antibiotic drug administration and a negative CSF culture result ranged from 15 minutes to 60 hours. Three of 15 patients whose CSF was sampled within 1 hour of antibiotic drug administration had a negative CSF culture result, and the infecting organism for each of these 3 cases was *N meningitidis*. Furthermore, CSF sterilization for each of the 37 cases of meningococcal meningitis occurred within 3 hours, suggesting that CSF sterilization, in some cases, occurs faster than previously believed. Therefore, based on the existing literature, our patient's clinical presentation, and the fact that he is an LCCD host, we believe that he likely had meningococcal meningitis. Our inability to detect the organism may relate to 1 or more factors, including low CSF bacterial concentration and previous antibiotic medication exposure. His complement deficiency state itself may have contributed to an abnormal CSF formula (elevated protein level and neutrophilic pleocytosis) and low CSF bacterial concentration, but no data exist on the expected CSF findings in LCCD hosts with bacterial meningitis.

Once an LCCD individual is identified, the clinician should focus on education and counseling.[51] Patients and their

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families should learn how to recognize and respond to the early signs and symptoms of meningococcal sepsis and meningitis. Patients with a good understanding of their condition will likely be able to live a normal life without the overriding fear of recurrent meningococcal infection. A medical identification bracelet should be ordered for the patient, and appropriate family members should be screened to see whether they are unrecognized LCCD hosts.[55] Select patients (and their families) may benefit from meeting with a qualified genetic counselor.

Prophylactic strategies, including long-term chemoprophylaxis and immunoprophylaxis, should also be considered for LCCD hosts. Because complement-deficient hosts develop meningococcal infection relatively infrequently, and because the interval between recurrences may be many years, it is difficult to determine the efficacy of such prophylactic strategies. Antibiotic prophylaxis (ie, penicillin injected monthly) has been used successfully in this situation,[56,57] but there are several downsides to this approach, including difficulties associated with committing a child or young adult to lifelong therapy and the prospect of selecting out for antibiotic-resistant strains. Meningococcal vaccination in LCCD hosts has also been investigated by numerous researchers.[19–21,54,58–60] The tetravalent meningococcal vaccine promotes antibody production to capsular serogroups A, C, Y, and W-135, but LCCD patients must be educated that the vaccine lacks protection against serogroup B infection and fails to fully compensate for the absent complement-mediated serum bactericidal activity. Our patient was fitted for a medical identification bracelet and was administered the tetravalent meningococcal vaccine, and he and his family were educated about meningococcal disease and vaccine efficacy. To date, he has had no evidence of recurrent infection.

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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 cluted 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.).

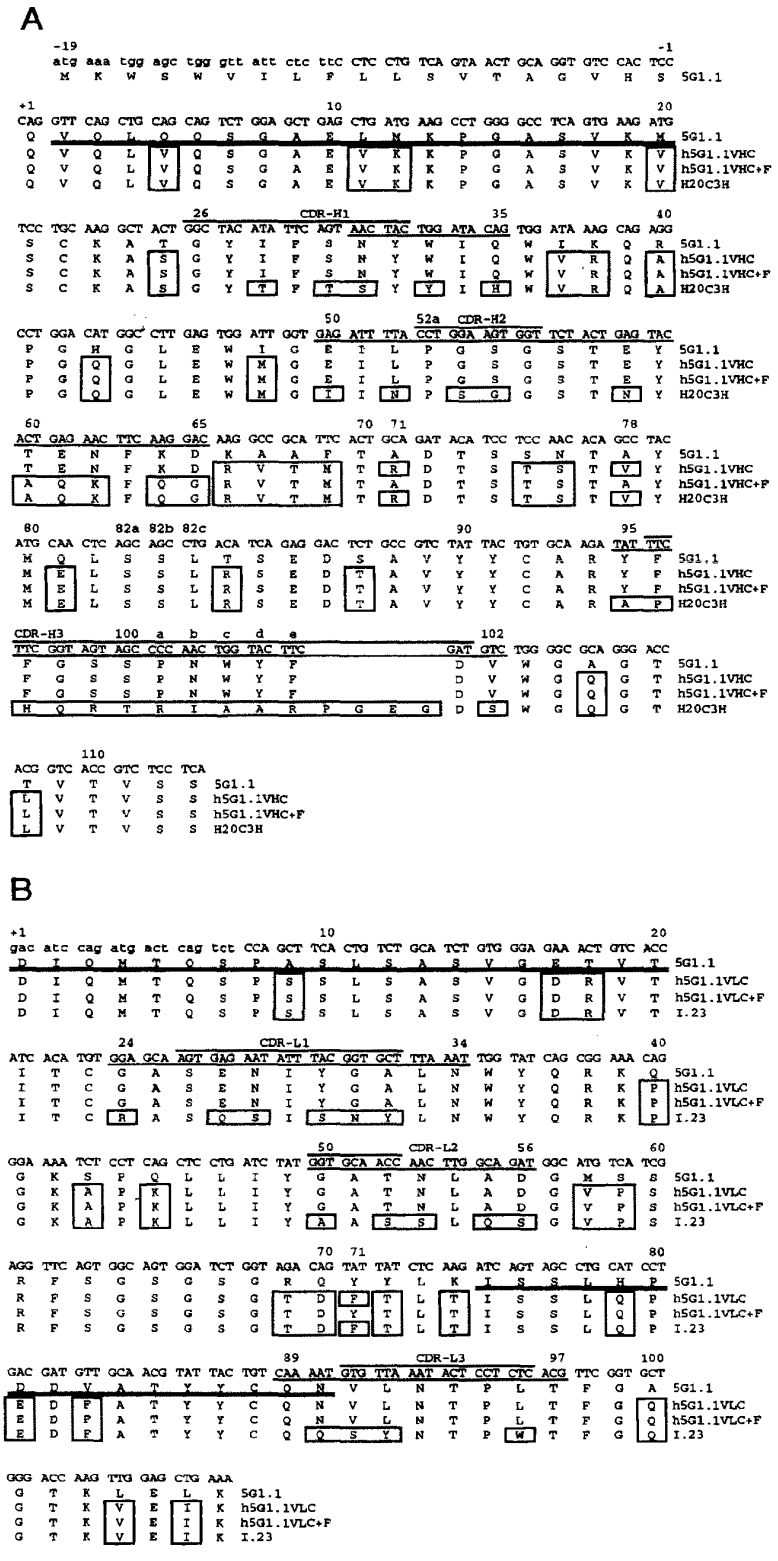
Construction of a humanized scFv

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

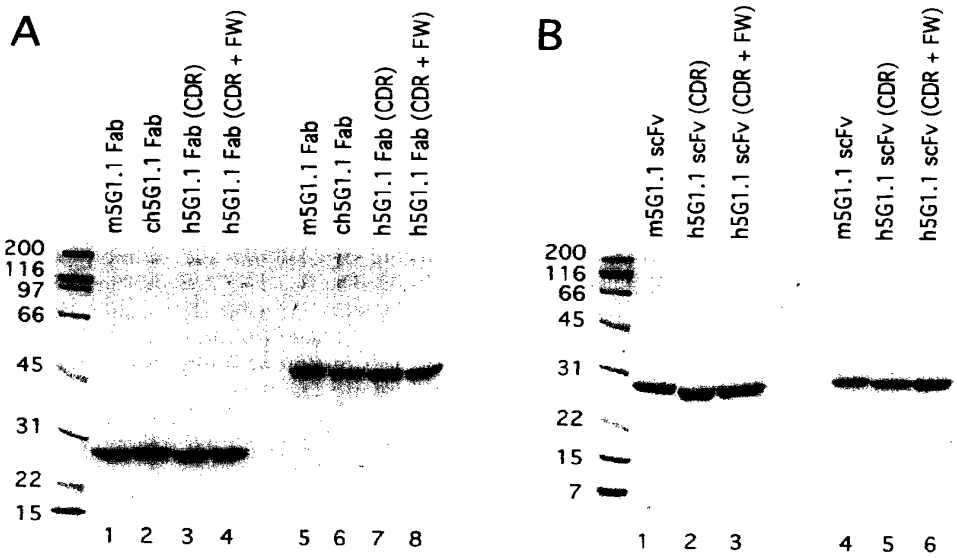


Fig. 2. SDS–PAGE analysis of purified recombinant proteins. (A) Fab fragments from the murine 5G1.1 mAb (m5G1.1 Fab) were produced by papain digestion of the m5G1.1 mAb followed by protein A chromatography to remove undigested antibody and Fc fragments. Recombinant chimeric Fab (ch5G1.1 Fab), CDR-grafted 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.

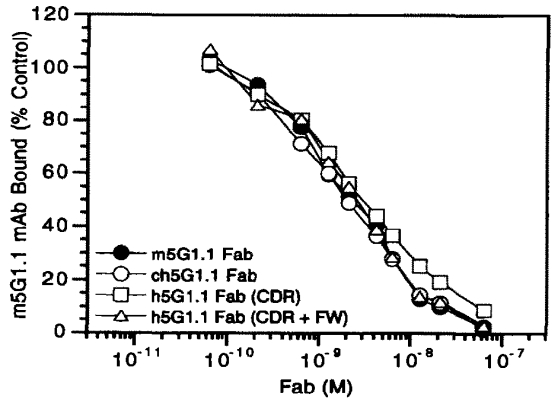


Fig. 3. Inhibition of 5G1.1 mAb binding to human C5 by recombinant Fab. 5G1.1 (0.2 nM) mAb plus the indicated concentrations of recombinant Fab were incubated together, for 2 hr at 37°C in microtiter plates coated with human C5. After washing, bound 5G1.1 mAb was detected using peroxidase-conjugated 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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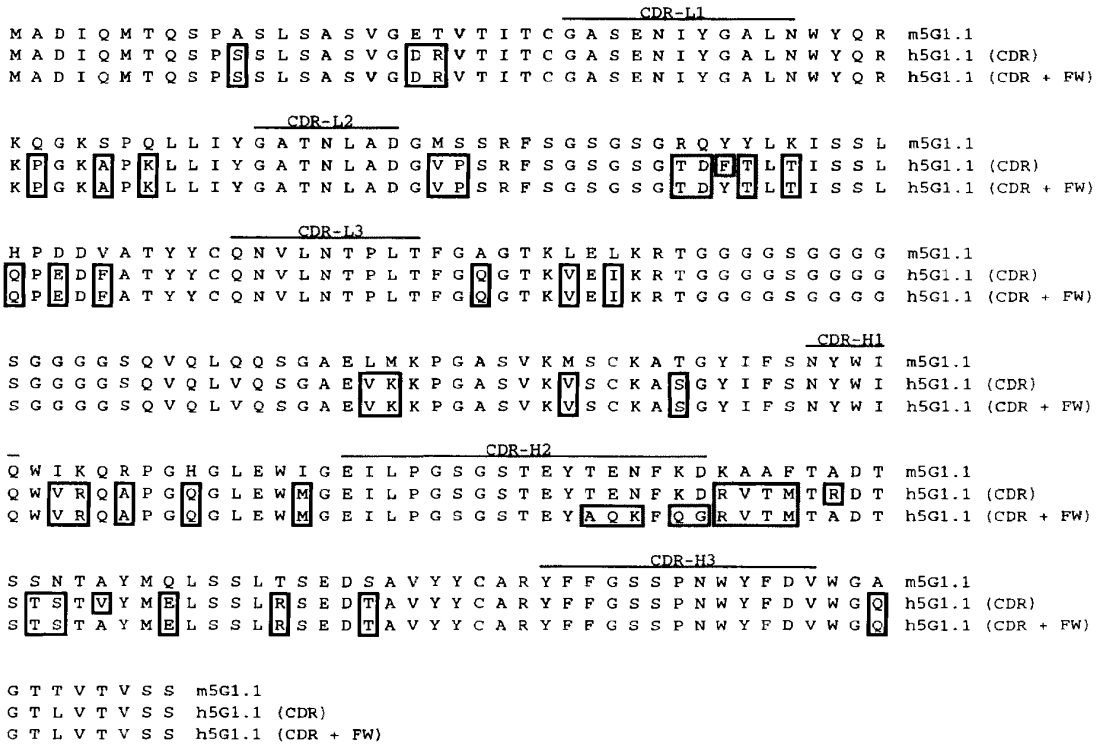


Fig. 4. Sequence of scFv molecules constructed using the 5G1.1 murine variable regions (m5G1.1) or humanized variable regions. The h5G1.1 (CDR) scFv contains the variable regions h5G1.1 VLC and h5G1.1 VHC, while the h5G1.1 scFv (CDR + FW) contains the variable regions h5G1.1 VLC+F and h5G1.1 VHC+F. The 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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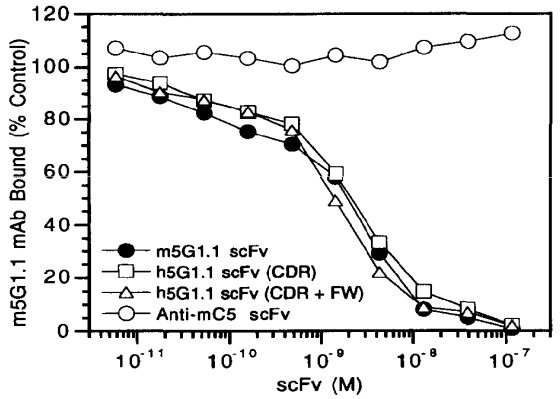


Fig. 5. Inhibition of 5G1.1 mAb binding to human C5 by recombinant scFv. 5G1.1 mAb (0.2 nM) plus the indicated concentrations of recombinant scFv were incubated together for 2 hr at 37°C, in microtiter plates coated directly with human C5. After washing, bound 5G1.1 mAb was detected using peroxidase-conjugated 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.

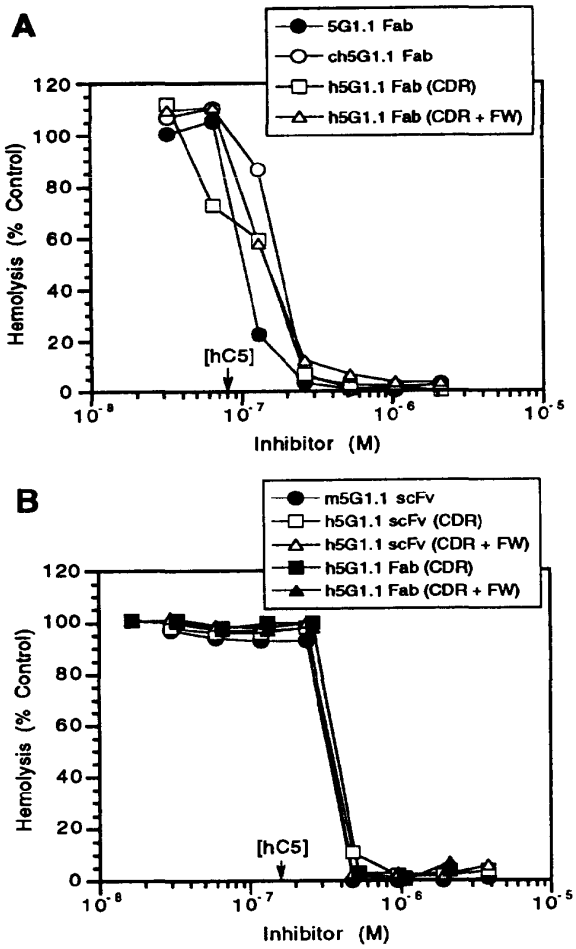


Fig. 6. Inhibition of chicken erythrocyte lysis by recombinant Fab and scFv. Twenty percent (A), or 40% (B), human serum in $GVBS^{++}$ was pre-incubated for 30 min at room temperature, with the indicated concentrations of competitors. Chicken erythrocytes, precoated with rabbit anti-chicken erythrocyte 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

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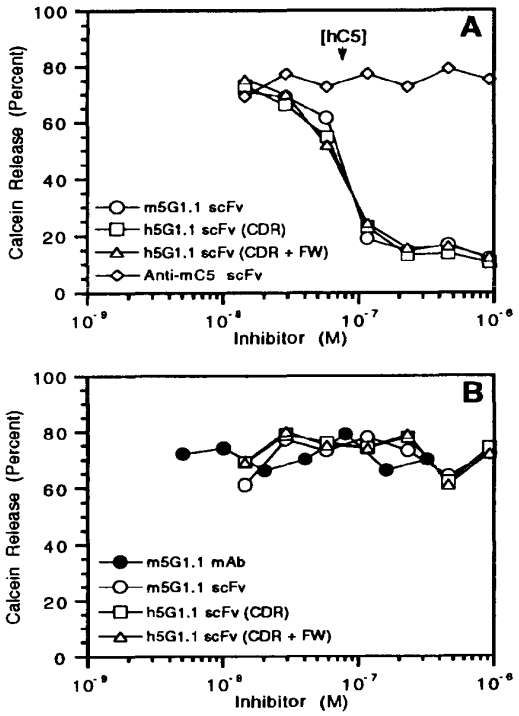


Fig. 7. Protection of porcine aortic endothelial cells (PAEC) from human serum complement by scFv. Calcein-labeled PAEC were incubated with HBSS containing 1% BSA and 0.85 mg/ml purified rabbit anti-PAEC polyclonal antibody for 30 min at 37°C. The cells were washed and incubated with HBSS containing 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.

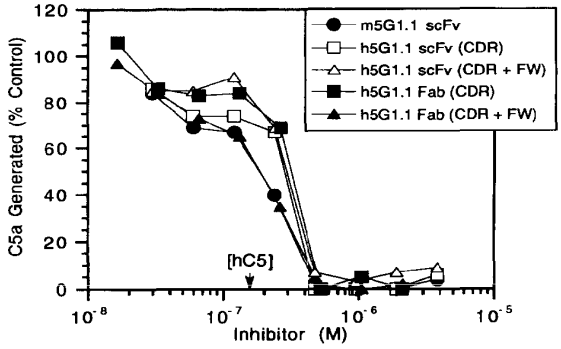


Fig. 8. Inhibition of C5a generation by recombinant Fab and scFv. Supernatants from chicken erythrocyte hemolytic assays performed using 40% human serum, as described in Fig. 5, were assayed for C5a content by ELISA. The C5a concentration obtained in the absence of inhibitor was defined as 100%. Data shown are from a representative experiment of several performed. The predicted hC5 concentration in 40% serum is indicated by the arrow.

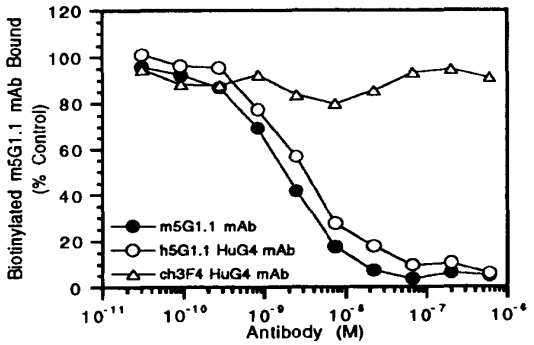


Fig. 9. Inhibition of 5G1.1 mAb binding to human C5 by recombinant humanized h5G1.1 HuG4 mAb. Biotinylated 5G1.1 mAb (0.2 nM), plus the indicated concentrations of unlabeled murine 5G1.1 mAb, recombinant h5G1.1 HuG4 mAb or a negative control recombinant HuG4 mAb (ch3F4 HuG4 mAb), were incubated together 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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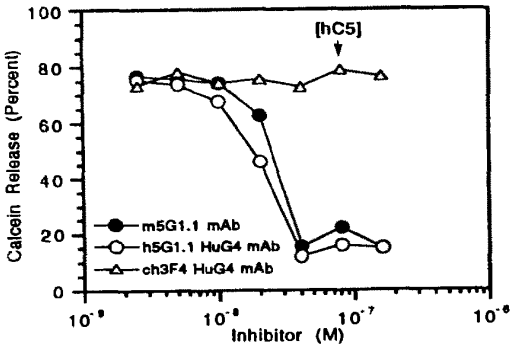


Fig. 10. Protection of porcine aortic endothelial cells from human serum complement by h5G1.1 HuG4 mAb. Calcein-labeled PAEC were incubated with HBSS containing 1% BSA and 0.85 mg/ml purified rabbit anti-PAEC polyclonal antibody for 30 min at 37°C. The cells were washed and incubated with HBSS containing 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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ORIGINAL ARTICLE

The Complement Inhibitor Eculizumab in Paroxysmal Nocturnal Hemoglobinuria

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ABSTRACT

BACKGROUND

We tested the safety and efficacy of eculizumab, a humanized monoclonal antibody against terminal complement protein C5 that inhibits terminal complement activation, in patients with paroxysmal nocturnal hemoglobinuria (PNH).

METHODS

We conducted a double-blind, randomized, placebo-controlled, multicenter, phase 3 trial. Patients 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 other week through week 26. The two primary end points were the stabilization of hemoglobin levels and the number of units of packed red cells transfused. Biochemical indicators of intravascular hemolysis and the patients' quality of life were also assessed.

RESULTS

Eighty-seven patients underwent randomization. Stabilization of hemoglobin levels in the absence of transfusions was achieved in 49% (21 of 43) of the patients assigned to eculizumab and none (0 of 44) of those assigned to placebo (P<0.001). 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<0.001). Eculizumab reduced intravascular hemolysis, as shown by the 85.8% lower median area under the curve for lactate dehydrogenase plotted against time (in days) in the eculizumab group, as compared with the placebo group (58,587 vs. 411,822 U per liter; P<0.001). Clinically significant improvements were also found in the quality of life, as measured by scores on the Functional Assessment of Chronic Illness Therapy-Fatigue instrument (P<0.001) and the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire. Of the 87 patients, 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.

CONCLUSIONS

Eculizumab is an effective therapy for PNH. (ClinicalTrials.gov number, NCT00122330.)

From Leeds General Infirmary, Leeds, United Kingdom (P.H., A.H.); National Heart, Lung, and Blood Institute, Bethesda, MD (N.S.Y.); Saarland University Medical School, Homburg-Saarland, Germany (J. Schubert); Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore (R.A.B.); Hôpital Saint-Louis and INSERM, Paris (G.S.); Radboud University Medical Center, Nijmegen, the Netherlands (P.M.); University Hospital of Essen, Essen, Germany (A.R.); Royal Melbourne Hospital, Parkville, Melbourne, Australia (J. Szer); St. George Hospital, London (M.O.E.); City of Hope National Medical Center and Beckman Research Institute, Duarte, CA (R.N.); St. James' Hospital, Trinity College Dublin, Dublin (P.B.); Federico II University, Naples (A.M.R.); the Institute of Transfusion Medicine, University Hospital, Ulm, Germany (H.S.); Cleveland Clinic Florida, Weston, FL (C.-L.F.); Taussig Cancer Center, Cleveland Clinic Foundation, Cleveland (J.M.); Alexion Pharmaceuticals, Cheshire, CT (S.A.R., C.F.M., R.P.R.); and Istituto Toscano Tumori, Florence, Italy (L.L.). 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@nhs.net.

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PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (PNH), an uncommon form of hemolytic anemia, results from the clonal expansion of hematopoietic stem cells that have somatic mutations in the X-linked gene *PIG-A*.[1,2] *PIG-A* mutations cause an early block in the synthesis of glycosylphosphatidylinositol (GPI) anchors, which tether many proteins to the cell surface. Consequently, the blood cells in patients with PNH have a partial deficiency (type II) or a complete deficiency (type III) of GPI-linked proteins.

Intravascular hemolysis is a prominent feature of PNH and is the consequence of the absence of the GPI-linked complement regulatory protein CD59.[3,4] CD59 blocks the formation of the terminal complement complex (also called the membrane-attack complex) on the cell surface, thereby preventing erythrocyte lysis and in vitro platelet activation.[5-8] Excessive or persistent intravascular hemolysis in patients with PNH causes anemia, hemoglobinuria, and complications related to the presence of plasma free hemoglobin, including thrombosis, abdominal pain, dysphagia, erectile dysfunction, and pulmonary hypertension.[9-12] Indeed, the symptoms in PNH are often disproportionate to the degree of anemia. Many patients with this disease are dependent on transfusions. Currently, there is no therapy that effectively reduces intravascular hemolysis or improves the symptoms in patients with PNH.

Eculizumab (Soliris, Alexion Pharmaceuticals) is a humanized monoclonal antibody directed against the terminal complement protein C5.[13] In a preliminary, 12-week, open-label clinical study involving 11 patients with PNH, eculizumab reduced intravascular hemolysis and the patients' transfusion requirements.[14] However, this two-center, uncontrolled study did not have a control group or predefined criteria for the administration of a transfusion, such as a predefined hemoglobin level at which transfusions were administered or a prespecified number of units of packed red cells for a given hemoglobin level.

We report the results of the phase 3 Transfusion Reduction Efficacy and Safety Clinical Investigation, a Randomized, Multicenter, Double-Blind, Placebo-Controlled, Using Eculizumab in Paroxysmal Nocturnal Hemoglobinuria (TRIUMPH) study, which investigated whether eculizumab stabilized hemoglobin levels and reduced transfusion requirements in 87 transfusion-dependent patients with PNH during 6 months of treatment. Intravascular hemolysis and the quality of life were also assessed.

METHODS

PATIENTS

The trial consisted of a 2-week screening period, an observation period of up to 3 months, and a 26-week treatment period. Patients 18 years of age or older who had received at least four transfusions during the previous 12 months were eligible. A PNH type III erythrocyte proportion of 10% or more, platelet counts of at least 100,000 per cubic millimeter, and lactate dehydrogenase levels that were at least 1.5 times the upper limit of the normal range were also required. Concomitant administration of erythropoietin, immunosuppressive drugs, corticosteroids, coumarins, low-molecular-weight heparins, iron supplements, and folic acid were permitted, provided that the doses were constant before and throughout the study. Because persons who have a genetic deficiency of terminal complement proteins have an increased risk of neisserial infections, all patients were vaccinated against *Neisseria meningitidis* with the use of locally approved vaccines. The protocol was approved by the institutional review board at each center, and all patients gave written informed consent.

Patients receiving transfusions who had a mean hemoglobin level greater than 10.5 g per deciliter before transfusion during the 12 months before entry into the study or who had received another investigational drug within 30 days before the first visit were excluded. Patients who had a complement deficiency, an active bacterial infection, or a history of meningococcal disease and those who had undergone bone marrow transplantation were also excluded.

An individualized transfusion algorithm was calculated for each patient on the basis of the history of transfusions during the previous 12 months; the written algorithm documented the number of units of packed red cells to be transfused for given hemoglobin values and served as a prospectively determined guide for transfusion during the observation and treatment periods. Before randomization, eligible patients were observed for up to 13 weeks. Patients who did not require a transfusion during the observation period were considered ineligible. A transfusion administered to a patient who had a hemoglobin

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level of 9 g per deciliter or less with symptoms or 7 g per deciliter or less with or without symptoms qualified the patient for the study (qualifying transfusion) and established the hemoglobin set point. This set point was required for the primary efficacy variable and was individualized for each patient.

STUDY DESIGN

Randomization was performed centrally in a 1:1 ratio without blocking and with stratification according to the number of units of packed red cells transfused during the past year; patients were assigned, by means of an interactive voice–response system, to receive either placebo or eculizumab within 10 days after the administration of the qualifying transfusion. Patients received infusions of 600 mg of eculizumab or placebo every week (±2 days) for 4 weeks, followed 1 week (±2 days) later by 900 mg of eculizumab or placebo, and then by a maintenance dose of 900 mg of eculizumab or placebo every 2 weeks (±2 days) through week 26.

CLINICAL EFFICACY

The two primary end points were the stabilization of hemoglobin levels, defined as a hemoglobin value that was maintained above the level at which the qualifying transfusion was administered, in the absence of transfusions during the 26-week treatment period, and the number of units of packed red cells transfused during that period. The trigger for the administration of transfusions during the study remained unchanged: patients received transfusions when they had symptoms resulting from anemia and their hemoglobin levels reached the individualized, predetermined set point. Prespecified secondary end points included transfusion independence; hemolysis, as measured by the lactate dehydrogenase value for the area under the curve from baseline to 26 weeks; and changes in the level of fatigue, as assessed from baseline to 26 weeks with the use of the Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT-Fatigue) instrument (scores can range from 0 to 52, with higher scores indicating improvement in fatigue).[15] Prespecified exploratory analyses included assessment of the quality of life with the use of the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire (EORTC QLQ-C30) (scores can range from 0 to 100, with higher scores on the global health status and functioning scales and lower scores on the symptom scales and single-item measures indicating improvement)[16]; changes in lactate dehydrogenase levels from baseline through week 26; and the presence of thrombosis. Other prespecified measurements included the pharmacokinetics, pharmacodynamics, and immunogenicity of eculizumab. The time to the first transfusion during the treatment period and the proportion of PNH type III blood cells were assessed.

SAFETY

Adverse events related to study infusions and vital signs (assessed at each of the 17 study visits during treatment), the results of biochemical analyses and blood counts (assessed at 9 visits), and findings on electrocardiograms (assessed at 3 visits) were documented. Adverse events were coded with the use of preferred terms from the Medical Dictionary for Regulatory Activities (MedDRA) (www.msso.org/MSSOWeb/index.htm) and tabulated as incidence rates in the two study groups.

STATISTICAL ANALYSIS

The planned sample size of 75 patients provided the study with a statistical power of 82%, at an alpha level of 0.05, to detect an increase of 35 percentage points (i.e., a change from 20% to 55%) in the rate of the stabilization of hemoglobin levels and a reduction in the median number of units of packed red cells transfused from 6 to 2 (±2). For the two primary end points, the analyses were performed according to the intention-to-treat principle with the use of data on all 87 patients who underwent randomization; stabilization of hemoglobin levels was analyzed with the use of Fisher's exact test, and the total number of units of packed red cells transfused was analyzed with the use of the Wilcoxon rank-sum test. To assess the effect of treatment on whether or not transfusions were required, Fisher's exact test was used. The log-rank test was used to compare the time to the first transfusion in the two groups. The area under the curve for lactate dehydrogenase was compared between the two groups with the use of the Wilcoxon rank-sum test.

Fatigue was assessed according to the scoring guidelines for the FACIT-Fatigue instrument.[17] The assessment of the quality of life was based on the EORTC QLQ-C30 scores and was conducted as described previously.[18] Changes in scores

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on the FACIT-Fatigue instrument and the EORTC QLQ-C30 instrument from baseline through week 26 were analyzed with the use of a mixed model, with baseline scores as the covariate, treatment and time as fixed effects, and the patient identifier as a random effect. Changes in the levels of lactate dehydrogenase, PNH type III erythrocytes, and hemoglobin from baseline through week 26 were analyzed with the use of the same mixed model. All reported P values are two-sided and were not adjusted for multiple analyses. The incidence rates of adverse events were compared with the use of Fisher's exact test. No interim analyses were performed, and blinding regarding the results was maintained until the end of the study.

The authors and the sponsor were jointly responsible for the trial design and the development of the protocol. Data were collected by an electronic case-report form with the use of InForm software (version 4.0, Phase Forward) and were analyzed by the sponsor. The decision to publish the trial data and final decisions on the content of the manuscript rested with Dr. Hillmen in consultation with the other authors. The manuscript was prepared by Dr. Hillmen, with substantial review and comments by the other authors. All authors had access to the primary data and take responsibility for the veracity and completeness of the data reported.

Table 1. **Baseline Characteristics of the Patients.**

Characteristic	Placebo Group (N=44)	Eculizumab Group (N=43)
Sex — no.		
Male	15	20
Female	29	23
Age — yr		
Median	35	41
Range	18–78	20–85
Duration of PNH — yr		
Median	9.2	4.3
Range	0.5–38.5	0.9–29.8
Reticulocyte counts — per mm^3		
Median	204,400	206,600
Range	45,400–556,200	40,200–570,400
History of aplastic anemia — no. (%)	12 (27)	6 (14)
History of myelodysplastic syndrome — no. (%)	0	2 (5)
History of thrombosis — no. (%)	8 (18)	9 (21)
Total no. of thrombotic events	11	16
Use of erythropoietin — no. (%)	0	3 (7)
Use of cyclosporine — no. (%)	1 (2)	1 (2)
Use of anticoagulant agents (coumarins or heparins) — no. (%)	11 (25)	21 (49)
Use of corticosteroids or androgenic steroids — no. (%)	12 (27)	12 (28)

RESULTS

PATIENTS' CHARACTERISTICS

Of a total of 115 patients with PNH who underwent screening, 87 (35 men and 52 women) at 34 sites in the United States, Canada, Europe, and Australia who received a qualifying transfusion, met the inclusion criteria and did not meet any of the exclusion criteria were randomly assigned to eculizumab (43 patients) or placebo (44 patients) between October 2004 and June 2005. At each of 16 sites one patient underwent randomization, at each of 6 sites two patients underwent randomization, and at each of 12 sites 3 or more patients underwent randomization. There were no significant differences in the baseline characteristics of the patients in the two groups (Table 1).

Of 87 patients who underwent randomization, 85 completed the trial (see the Supplementary Appendix, available with the full text of this article at www.nejm.org). Two patients in the eculizumab group did not complete the trial, one because traveling to the study center was inconvenient and the other because of pregnancy; these patients were included in the analyses. Ten patients in the placebo group discontinued infusions because of a perceived lack of efficacy but remained in the study for monitoring, as prespecified in the protocol, and were included in the analyses.

PHARMACOKINETICS AND PHARMACODYNAMICS

In 42 of 43 patients in the eculizumab group, a 900-mg dose of eculizumab every 2 weeks (±2 days) completely blocked serum hemolytic activity, as assessed by a presensitized erythrocyte hemolytic assay,[14] throughout the study period. In one patient, therapeutic trough levels of eculizumab were not maintained.

EFFECT ON HEMOLYSIS

The effect of eculizumab on chronic intravascular hemolysis was demonstrated by an immediate

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(1 week) and sustained decrease in lactate dehydrogenase levels (Fig. 1A). In the eculizumab group, the mean (±SE) lactate dehydrogenase level decreased from 2199.7±157.7 U per liter at baseline to 327.3±67.6 U per liter at 26 weeks, whereas in the placebo group the levels remained elevated, with values of 2258.0±154.8 U per liter at baseline and 2418.9±140.3 U per liter at 26

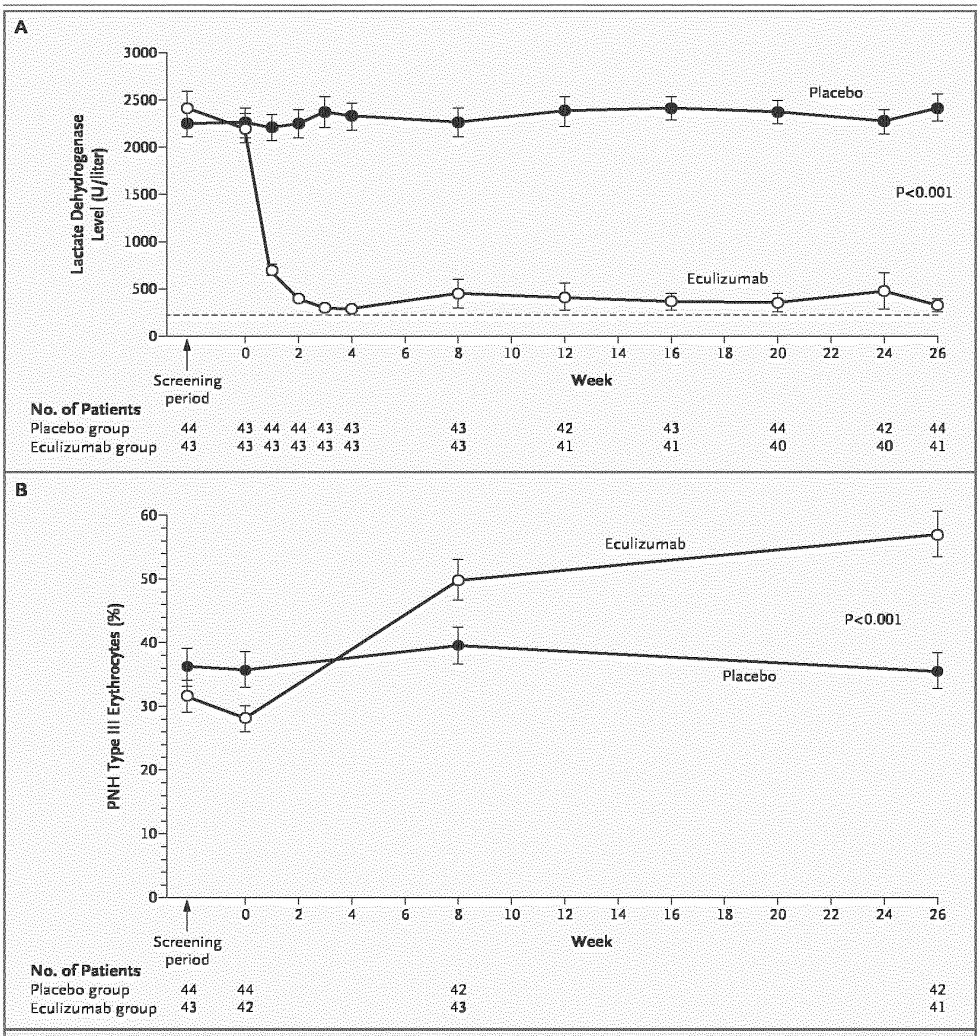


Figure 1. **Levels of Lactate Dehydrogenase and PNH Type III Erythrocytes during Treatment with Eculizumab.**

Panel A shows the degree of intravascular hemolysis according to the mean levels of lactate dehydrogenase from baseline (week 0) to week 26 in the two study groups. The dashed line indicates the upper limit of the normal range for lactate dehydrogenase (normal range, 103 to 223 U per liter). In the eculizumab group the mean level of lactate dehydrogenase was reduced to just above the upper limit of the normal range at week 26; of 41 patients in this group who completed the study, 15 had levels within the normal range. In the placebo group, all patients had levels at least five times above the upper limit of normal at week 26. Panel B shows the mean proportion of PNH type III erythrocytes in patients in the two groups. Screening occurred up to 3 months before week 0. P values are from a mixed analysis-of-covariance model from baseline through week 26. I bars indicate the standard error.

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weeks (P<0.001). The median value of the areas under the curve for lactate dehydrogenase plotted against time (in days) was 85.8% lower in the eculizumab group than in the placebo group (58,587 vs. 411,822 U per liter; P<0.001). A second biochemical measure of hemolysis, the serum level of aspartate aminotransferase, also showed significant improvement with eculizumab, as compared with placebo (data not shown).

The reduction in intravascular hemolysis in the eculizumab group resulted in an increase in PNH type III erythrocytes (Fig. 1B) from a mean of 28.1±2.0% at baseline to 56.9±3.6% at week 26. The proportion of PNH type III erythrocytes in patients in the placebo group remained constant (35.7±2.8% before treatment and 35.5±2.8% at 26 weeks, P<0.001 for the comparison with the eculizumab group and the placebo group). The proportion of PNH type III granulocytes and monocytes did not change significantly between the two groups (data not shown).

CLINICAL EFFICACY

Primary End Points

The two primary efficacy end points were the stabilization of hemoglobin levels and the number of units of packed red cells transfused. At the end of the treatment period, 49% of patients in the eculizumab group (21 of 43) had levels of hemoglobin that remained above the prespecified set point (median, 7.7 g per deciliter for both groups) in the absence of transfusions, whereas stabilization of hemoglobin levels did not occur in any patient in the placebo group (P<0.001) (Table 2). By week 26, the median number of units of packed red cells transfused per patient was 0 in the eculizumab group and 10 in the placebo group (P<0.001), whereas the mean number of units of packed red cells transfused was 3.0±0.7 and 11.0±0.8, respectively. In the 6-month period before the study, the median number of units of packed red cells transfused per patient was 9.0 in the eculizumab cohort and 8.5 in the placebo cohort, and the mean number of units of packed red cells transfused was 9.6±0.6 and 9.7±0.7, respectively. The mean hemoglobin levels changed from 10.0±0.2 g per deciliter and 9.7±0.2 g per deciliter in the eculizumab group and the placebo group, respectively, at baseline to 10.1±0.2 g per deciliter and 8.9±0.2 g per deciliter, respectively, at week 26 (P<0.001, by mixed-model analysis).

The median time to the first transfusion was significantly longer in eculizumab-treated patients than in patients who received placebo (P<0.001) (Fig. 2). Transfusion independence was achieved in 51% of patients in the eculizumab group (22 of 43) and 0% of those in the placebo group (0 of 44, P<0.001). By week 26, the total number of units of packed red cells transfused was 131 in the eculizumab group and 482 in the placebo group (Table 2). By contrast, during the 6 months before the study, the total number of units transfused was 413 in the eculizumab group and 417 in the placebo group.

Table 2. Stabilization of Hemoglobin Levels and the Number of Units of Packed Red Cells Transfused during Treatment.*

Primary End Point	Before Treatment†		During Treatment		P Value
	Placebo Group	Eculizumab Group	Placebo Group	Eculizumab Group	
Patients with stabilized hemoglobin levels (%)	NA	NA	0	49	<0.001‡
Packed red cells transfused (units/patient)					
Median	8.5	9.0	10	0	<0.001§
Interquartile range	7–12.5	6–12	6–16	0–6	
Mean	9.7±0.7	9.6±0.6	11.0±0.8	3.0±0.7	
Total	417	413	482	131	

* Plus–minus values are means ±SE. NA denotes not applicable.
† Transfusion data obtained during 12 months before treatment were normalized to a value equivalent to the value for a 6-month period.
‡ The P value is for the comparison between groups during treatment, calculated with the use of a two-tailed Fisher's exact test.
§ The P value is for the comparison between groups during treatment, calculated with the use of the Wilcoxon rank-sum test.

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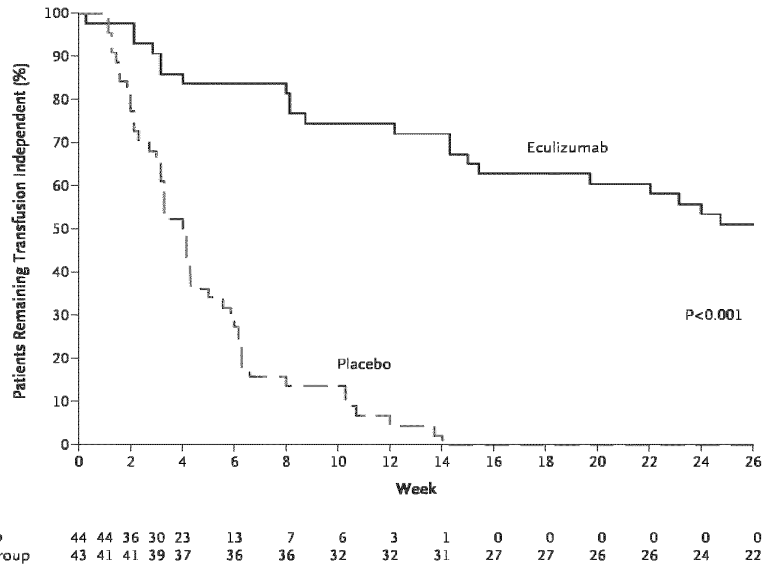


Figure 2. **Kaplan–Meier Curves for the Time to the First Transfusion during Treatment.**
The P value for the comparison of times to the first transfusion between the two groups was calculated by the log-rank test.

Quality of Life

Assessments of the quality of life were performed with the use of two instruments, the FACIT-Fatigue instrument and the EORTC QLQ-C30 instrument. Patients in the eculizumab group had a mean increase (improvement) in scores on the FACIT-Fatigue instrument of 6.4±1.2 points from baseline to week 26, whereas in the placebo group the mean score decreased by 4.0±1.7 points during this period, for a total difference between the two groups of 10.4 points (Fig. 3). A mixed-model analysis of covariance was performed that showed a significant difference between the two groups ($P<0.001$).

With respect to the EORTC QLQ-C30 instrument, the eculizumab group had significant improvements in scores on the scale for global health status, on all five scales for functioning, on two of three symptom scales, and on three of six single-item measures, as compared with the placebo group ($P\leq0.01$ for each scale and measure) (Table 3).

SAFETY

No patients died during the study. Serious adverse events were reported in 13 patients: 4 in the eculizumab group and 9 in the placebo group (Table 4). No serious adverse events 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. The number of headaches that occurred was similar in the two groups after the first 2 weeks of therapy. There were no significant differences in the incidence rates between the two groups for any reported adverse event. A single thrombosis occurred in a patient in the placebo group.

One patient in each of the two groups had detectable levels of antibodies against eculizumab. The levels were low, were detected at a single visit, and in the patient receiving eculizumab, the antibodies did not affect complement inhibition.

DISCUSSION

Patients with PNH have chronic intravascular hemolysis with acute exacerbations. Anemia and the

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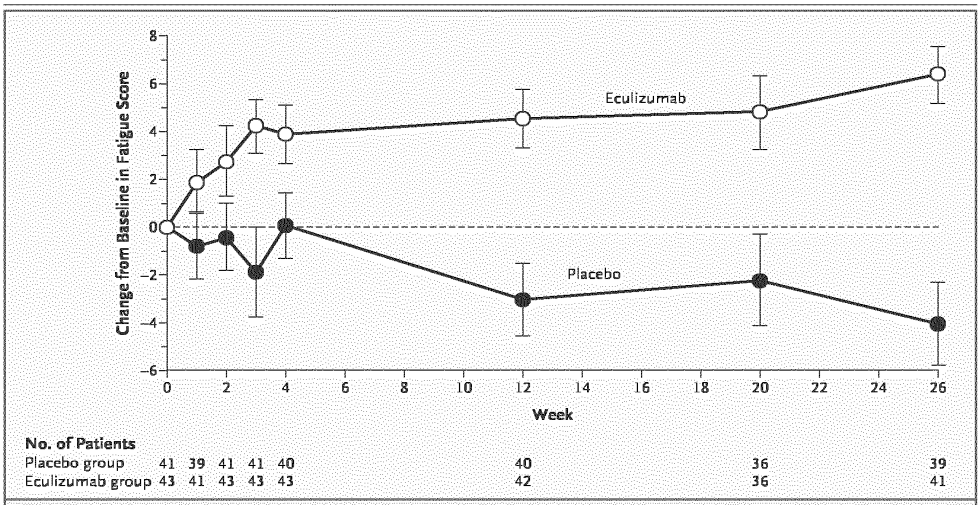


Figure 3. Change in Fatigue Scores from Baseline to Week 26.

Week 0 (baseline) represents the end of the observation period, within 10 days after the patient's receipt of the qualifying blood transfusion. Fatigue scores are therefore higher (indicating less fatigue) at week 0 than at the initial screening visits (data not shown). Values for the change from baseline (dashed line) represent means. A positive change from baseline indicates an improvement in fatigue and a negative change from baseline indicates a worsening in fatigue. I bars indicate the standard error.

need for transfusions to sustain hemoglobin levels occur frequently, as does deterioration of the patient's quality of life. In this study, in approximately half the patients treated with eculizumab, the end points of stabilization of hemoglobin levels and transfusion independence were reached, whereas none of the patients in the placebo group reached either of these end points. The median time to the first transfusion was 4 weeks in the placebo group and more than 6 months in the eculizumab group. The overall rate of transfusion was reduced by 73% in the eculizumab group. Even among patients receiving eculizumab in whom transfusion independence was not reached, the number of units of packed red cells transfused was reduced by 44%, as compared with patients in the placebo group (data not shown).

Intravascular hemolysis is central to the occurrence of serious coexisting conditions in patients with PNH and contributes to the risk of death among these patients.[9,12] Lactate dehydrogenase, a biochemical marker of hemolysis, was immediately reduced from approximately 10 times the upper limit of the normal range to normal levels or to just above normal levels in all patients in the eculizumab group. Residual low-level hemolysis in some patients despite terminal-complement blockade may be caused by an inherent decrease in the survival of PNH type III erythrocytes[19] or may be due to the fact that these cells are opsonized with C3b, which mediates extravascular clearance through the reticuloendothelial system.

Before treatment with eculizumab, the hemoglobin levels were maintained by transfusion. Therefore, the stabilization of hemoglobin levels with a concomitant cessation of or reduction in the number of transfusions indicates an increase in endogenous erythrocyte mass. The reduction in hemolysis with eculizumab results in a new steady-state hemoglobin level, as determined by a balance of the underlying bone marrow dysfunction, the increased half-life of PNH erythrocytes because of eculizumab therapy, and the new level of transfusions (if any) required.

For most patients with PNH, the quality of life is impaired, and the impairment has been attributed not only to anemia but also to excessive in-

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Table 3. Change in the Quality of Life during Treatment.*

Scale	Mean Change in Score from Baseline to Week 26†		Absolute Difference	P Value‡
	Placebo Group	Eculizumab Group		
Global health status scale	−8.5	10.9	19.4	<0.001
Functioning scales				
Role	−6.9	17.9	24.8	<0.001
Social	2.0	16.7	14.7	0.003
Cognitive	−6.1	7.9	14.0	0.002
Physical	−3.5	9.4	12.9	<0.001
Emotional	−3.7	7.5	11.2	0.008
Symptom scales				
Fatigue	10.0	−16.9	26.9	<0.001
Pain	5.3	−12.3	17.6	0.002
Nausea and vomiting	2.8	−0.4	3.2	0.06
Single-item measures				
Dyspnea	8.9	−7.9	16.8	<0.001
Loss of appetite	3.3	−10.3	13.6	<0.001
Insomnia	4.9	−7.9	12.8	0.01
Financial difficulties	0.0	−10.3	10.3	0.19
Constipation	0.0	−6.3	6.3	0.20
Diarrhea	5.7	4.8	0.9	0.15

* The quality of life was assessed with the EORTC QLQ-C30 instrument.
† A positive value for a score on the scales for global health status and functioning indicates improvement, whereas a negative value for a score on the symptom scales and for a score on the single-item measures indicates improvement.
‡ P values are from a mixed model, with baseline scores as the covariate, treatment and time as fixed effects, and the patient identifier as a random effect.

travascular hemolysis and the scavenging of nitric oxide by cell-free hemoglobin.[9-11] In this study, the reduction in intravascular hemolysis with eculizumab, as compared with placebo, was associated with a significant improvement in fatigue, as assessed by scores on the FACIT-Fatigue instrument. Eculizumab increased the baseline score for fatigue by 6.4 points. A change of three or more points in scores on this instrument represents a clinically important difference.[20] Improvement with eculizumab in the fatigue component of the EORTC QLQ-C30 instrument provides additional evidence for the benefit shown by scores on the FACIT-Fatigue instrument. These improvements with eculizumab occurred without complete resolution of the anemia, providing further evidence of the contribution of hemolysis, in contrast to anemia, to the diminishing quality of life of patients with PNH. Clinical assessment of additional symptoms related to the quality of life of such patients, including abdominal pain, dysphagia, and erectile dysfunction, have also been reported to improve during eculizumab therapy.[21]

There were no deaths during the study, and only a single thrombotic event occurred in a patient in the placebo group. There were four serious adverse events in the eculizumab group and nine in the placebo group; all these patients recovered. The issue of possible protection against the risk of thrombosis through terminal complement inhibition with eculizumab is being evaluated in ongoing clinical studies of PNH. All 85 patients who completed the study elected to receive eculizumab in an open-label extension study.

The results of this randomized, double-blind, controlled study show that terminal complement inhibition with eculizumab reduces intravascular

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Table 4. Adverse Events.*

Adverse Event	Placebo Group (N=44)	Eculizumab Group (N=43)
	no. (%)	
Total no. of serious adverse events	9 (20)	4 (9)
Exacerbation of PNH	3 (7)	1 (2)
Renal colic	0	1 (2)
Lumbar- or sacral-disk prolapse	0	1 (2)
α-Hemolytic streptococcal bacteremia	0	1 (2)
Central-line and urinary tract infections	1 (2)	0
Upper respiratory tract infection	1 (2)	0
Probable viral infection	1 (2)	0
Neutropenia	1 (2)	0
Cellulitis, folliculitis, and neutropenia	1 (2)	0
Anemia and pyrexia	1 (2)	0
Most frequent adverse events†		
Headache‡	12 (27)	19 (44)
Nasopharyngitis	8 (18)	10 (23)
Upper respiratory tract infection	10 (23)	6 (14)
Back pain	4 (9)	8 (19)
Nausea	5 (11)	7 (16)
Cough	4 (9)	5 (12)
Diarrhea	5 (11)	4 (9)
Arthralgia	5 (11)	3 (7)
Abdominal pain	5 (11)	2 (5)
Dizziness	5 (11)	2 (5)
Vomiting	5 (11)	2 (5)
Fatigue	1 (2)	5 (12)
Viral infection	5 (11)	1 (2)

* Adverse events were coded with the use of preferred terms from the MedDRA.
† The event occurred in at least 10% of patients in either group.
‡ After the first 2 weeks of treatment, 10 patients (23%) receiving placebo and 9 patients (21%) receiving eculizumab had headache.

hemolysis, reduces or eliminates the need for transfusion, and improves anemia, fatigue, and the quality of life in patients with PNH. The data provide support for the central role of intravascular hemolysis in the pathogenesis of the disease and indicate that eculizumab is an effective treatment in patients with PNH.

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APPENDIX

In addition to the authors, the following investigators and institutions participated in the TRIUMPH study: **Australia** — Princess Alexandra Hospital, Woolloongabba: A. Mills; Queen Elizabeth Hospital, Woodville South: J. Norman; Royal Melbourne Hospital, Parkville; Royal Perth Hospital, Perth, WA: R. Herrmann; **Belgium** — St. Luc University Hospital, UCL, Brussels: E. Van Den Neste; **Canada** — University of Alberta, Cross Cancer Institute, Edmonton, AB.: L. Larratt, A. Turner, M.A. Hamilton; **Germany** — Universitätsklinikum Essen, Essen: U. Dührsen; Medizinische Hochschule Hanover, Hanover: A. Ganser; Universitätsklinik Greifswald, Greifswald: M. Montemurro; Institut für Klinische Transfusionsmedizin and Immungenetik, University Hospital Ulm, Ulm; Saarland University Medical School, Hamburg; **France** — Hospital de l'Hotel-Dieu, Paris: B. Rio; Hospital St. Louis and INSERM, Paris; **Ireland** — St. James Hospital, Dublin; **Italy** — Ospedale San Martino, Genoa: A. Bacigalupo; Azienda-Ospedaliera Universitaria Careggi, Florence: E. Antonioli, G. Gianfaldoni, F. Mannelli, A. Bosi; Ospedale San Bortolo, Vicenza: F. Rodeghiero; Federico II University, Naples: B. Rotoli, F. Alfinito; Ospedale Maggiore di Milano, Milan: A. Zanella, C. Boschetti; Istituto Toscana Tumori, Florence; **the Netherlands** — Radboud University Medical Center, Nijmegen; **Sweden** — Lund University Hospital, Lund: P.-G. Nillson; Umea University Hospital, Umea: A. Wahlin; Stockholm South Hospital, Stockholm: J. Samuelsson, L.G. Lundberg, P. Andersson; **United Kingdom** — St. George's Hospital, London; Leeds General Infirmary, Leeds; Belfast City Hospital, Belfast: M.F. McMullin; **United States** — Washington University School of Medicine, St. Louis: M. Bessler, L. Andritsos, M. Blinder, S. Devine; Johns Hopkins University Medical Center, Baltimore; Memorial Sloan-Kettering Cancer Center, New York: H. Castro-Malaspina, D. Araten; Stanford University Medical Center, Stanford, CA: S. Coutre; Duke University Medical Center, Durham, NC: C. de Castro III; Cleveland Clinic Florida, Weston, FL: E. Stone; University of Pennsylvania, Philadelphia: B. Konkle; Massachusetts General Hospital, Boston: D. Kuter; Cleveland Clinic Foundation, Cleveland: A. Lichtin; New York University Clinical Cancer Center, New York: T. Moskovits, B.G. Raphael, E. Amorosi, K.B. Hymes, P. Cook; City of Hope National Medical Center, Duarte, CA; Indiana University Cancer Center, Indianapolis: R. Nelson; University of California at Los Angeles, Los Angeles: R. Paquette; Hartford Hospital, Hartford, CT: R. Siegel; National Heart, Lung, and Blood Institute, Bethesda, MD: B. Savani.

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HUMANIZED PORCINE VCAM-SPECIFIC MONOCLONAL ANTIBODIES WITH CHIMERIC IgG2/G4 CONSTANT REGIONS BLOCK HUMAN LEUKOCYTE BINDING TO PORCINE ENDOTHELIAL CELLS

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Abstract—Discordant xenografts surviving the initial hyperacute rejection phase may be subject to cellular rejection processes mediated by infiltrating leukocytes including T cells, NK cells and monocytes. The stable adhesion of these cell types to endothelial cells is due to the molecular interaction of the integrins VLA-4 and LFA-1 with their ligands vascular cell adhesion molecule (VCAM) and ICAM-1 present on the endothelial cells. Human VLA-4 binds to porcine VCAM, and blocking mAbs specific for porcine VCAM have been developed. We have localized the epitope of the anti-porcine VCAM blocking mAbs 2A2 and 3F4 to domains 1 and 2, respectively. Humanized antibodies (IgG4 isotype) were constructed from these anti-porcine VCAM antibodies and demonstrated to inhibit adhesion of Ramos, Jurkat and YT cells, as well as purified resting and activated human T cells, to porcine aortic endothelial cells (PAEC). These cell types express both LFA-1 as well as VLA-4, suggesting blockade of human VLA-4 interaction with porcine VCAM may alone be sufficient to significantly impair adhesion of human leukocytes to porcine endothelial cells. The chimeric anti-porcine VCAM (pVCAM) HuG4 antibodies promoted increased adhesion of Fc receptor (FcR) positive cells such as U937 monocytic cells to PAEC. In contrast, chimeric anti-porcine VCAM antibodies created using the CH1 and hinge region from human IgG2 and the CH2 and CH3 regions from human IgG4 (HuG2/G4 antibodies) inhibited binding of FcR positive cells to PAEC. These chimeric anti-pVCAM antibodies should allow delineation of the *in vivo* role of VLA-4/VCAM interaction in porcine-to-primate xenotransplants. Further, the design of the HuG2/G4 antibodies should render them efficacious in multiple settings requiring elimination of FcR binding. © 1997 Elsevier Science Ltd.

Key words: adhesion molecules, antibodies, Fc receptors, inflammation, xenotransplantation.

INTRODUCTION

Although much progress has been made in elucidating the molecular mechanisms of hyperacute rejection of xenotransplants (reviewed in Somerville and D'Apice, 1993), the mechanisms of cell mediated rejection of xenografts remain unclear. The cellular infiltrate observed 3 days following transplantation of guinea pig hearts into complement (C)-depleted rats is comprised predominantly of macrophages and natural killer cells, with low numbers of T lymphocytes present (Blakely *et al.*, 1994). Similarly, the cellular infiltrate present 6 days after transplantation of porcine fetal islet clusters into rats is comprised predominantly of macrophages with low numbers of T lymphocytes present primarily at the periphery of the graft (Wallgren *et al.*, 1995). *In vitro* porcine aortic endothelial cells (PAEC) as well as antigen presenting peripheral blood cells can directly activate human CD4+ and CD8+ T lymphocytes (Rollins *et al.*, 1994*a*; Murray *et al.*, 1994; Yamada *et al.*, 1995). Thus the initial phases of cellular rejection of xenotransplants are likely to involve binding and activation of multiple types of leukocytes including not only T lymphocytes but also natural killer cells and monocytes.

The stable adhesion of lymphocytes, natural killer cells, and monocytes to endothelial cells is mediated predominantly by the interaction of the integrins LFA-1 (CD11a/CD18) and VLA-4 (CD49d/CD29) with their cognate ligands ICAM-1 and VCAM-1 on endothelial cells (Allavena *et al.*, 1991; Jonjic *et al.*, 1992). For both monocytes (Luscinskas *et al.*, 1994) and T lymphocytes (Alon *et al.*, 1995; Luscinskas *et al.*, 1995) the binding of VLA-4 to VCAM is an essential component of the *in vitro* adhesive interactions required for stable arrest under

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conditions of flow. *In vitro*, the transmigration of NK cells and monocytes across human umbilical vein endothelial cell (HUVEC) monolayers also utilizes both LFA-1/ICAM-1- and VLA-4/VCAM-dependent pathways (Bianchi *et al.*, 1993; Meershaert and Furie, 1994; Chuluyan *et al.*, 1995). *In vivo*, the interaction of LFA-1 with ICAM-1 may not be completely essential for extravasation of lymphocytes or monocytes since both of these cell types are present in inflammatory infiltrates of leukocyte adhesion deficiency patients who have a genetic deficiency in CD18 and thus lack integrins such as LFA-1 (Hawkins *et al.*, 1992). Finally, numerous studies have demonstrated the potential of blocking antibodies directed against LFA-1 (Issekutz, 1992; Scheynius *et al.*, 1993), VLA-4 (Issekutz, 1991; Yednock *et al.*, 1992; Chisholm *et al.*, 1993), ICAM-1 (Zeng *et al.*, 1994), or VCAM (Orosz *et al.*, 1993; Silber *et al.*, 1995) to inhibit the accumulation of lymphocytes at inflammatory sites in animals.

For xenogeneic cellular interactions it is not yet fully defined which adhesion molecule interactions will function across species barriers. It is likely that at least some xenogeneic receptor–ligand interactions will be nonfunctional. For example, mouse LFA-1 does not bind to human ICAM-1 (Johnston *et al.*, 1990). To explore the molecular basis of adhesive interactions between distinct classes of adhesion molecules on human and porcine cells we have cloned porcine E-selectin and VCAM-1 and have demonstrated that both are capable of mediating the adhesion of human leukocytes (Rollins *et al.*, 1994*a*; Mueller *et al.*, 1995). Further, we have developed murine monoclonal antibodies (mAbs) specific for porcine VCAM which block binding of human VLA-4 to porcine VCAM (Mueller *et al.*, 1995). The functional blocking activity of these antibodies suggests that they will be useful in suppressing the cellular response to a porcine xenograft. However, murine mAbs are not ideally suited for use in primates due to their short half-life and high immunogenicity (Khazaeli *et al.*, 1994). Here we have utilized antibody engineering strategies to create humanized anti-porcine VCAM antibodies that block adhesion of human natural killer cells, monocytes, and T lymphocytes to activated PAEC. Furthermore, we show the choice of human isotype has critical implications for the potential therapeutic use of humanized antibodies in xenotransplantation.

MATERIALS AND METHODS

Cells

Ramos, Jurkat and U-937 cells were obtained from the American Type Culture Collection, YT cells were the gift of Dr J. M. Wang (National Cancer Institute, Frederick, MD, U.S.A.), porcine aortic endothelial cells (PAEC) were obtained from Cell Systems (Kirkland, WA, U.S.A.) and 293-EBNA cells were obtained from Invitrogen (San Diego, CA, U.S.A.). U-937 cells were maintained in RPMI 1640 supplemented with 15% heat-inactivated FBS and 2 mM L-glutamine. Ramos and Jurkat cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 20 mM HEPES, 50 μM β-mercaptoethanol, 100 units/ml penicillin and 100 μg/ml streptomycin. YT were maintained in RPMI 1640 containing 10% heat-inactivated FBS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. PAEC were maintained in medium A consisting of M199 supplemented with 20% heat-inactivated FBS, 0.05 mg/ml ECGF (Biomedical Technologies, Stoughton, MA, U.S.A.), 20 mM HEPES and 0.1 mg/ml heparin, and were used within three passages. 293-EBNA cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and 250 μg/ml G418. Human T cells were purified as described previously (Mueller *et al.*, 1995).

Production of VCAM/Ig fusions

A plasmid containing the human IgG1 Fc region was obtained from Dr M. J. Lenardo (NIH, Bethesda, MD, U.S.A.). DNA fragments containing various domains of human or porcine VCAM were obtained by PCR amplification of appropriate templates and joined in frame to the human IgG1 Fc coding region using standard molecular biology techniques. The cassettes encoding the fusion proteins were ligated into the expression plasmid pAPEX-3 (Evans *et al.*, 1995*a*). VCAM/Ig fusion proteins were produced by transfection of 293-EBNA cells as described previously (Evans *et al.*, 1995*a*). Three days following transfection the media were harvested, centrifuged to remove debris, and stored at 4°C. For ELISA, PolySorp 96-well microtiter plates (Nunc, Naperville, IL, U.S.A.) were coated overnight at 4°C with 100 ng/well goat anti-human IgG Fc (Sigma, St. Louis, MO, U.S.A.) in 0.1 M carbonate buffer, pH 9.6. The plates were then washed three times with PBS supplemented with 0.5% Tween 20 and incubated with blocking buffer (PBS supplemented with 0.05% Tween 20) at 37°C for 1 hr. After three further washes, various dilutions of conditioned media in blocking buffer were added and incubated at 37°C for 1 hr. Following three further washes, 2A2 or 3F4 mAb in blocking buffer was added and incubated for 1 hr at 37°C. After additional washing, peroxidase-conjugated goat anti-mouse IgG Fc (Sigma) diluted 1:2000 in blocking buffer was added and incubated at 37°C for 1 hr. After three final washes the plate was developed for 15 min with 0.4 mg/ml *o*-phenylenediamine dihydrochloride (Sigma). Reactions were stopped by addition of 1 M sulfuric acid. Quantitation was performed by measuring absorbance at 490 nm using a Bio-Rad model 3550 plate reader.

Production of chimeric antibodies

Variable regions were cloned from the 2A2 and 3F4 hybridomas by PCR amplification as described previously (Evans *et al.*, 1995*a*, 1995*b*). These sequences have been submitted to the EMBL/GenBank/DDBL under accession numbers U78799–U78802. The variable regions were joined to genomic DNA cassettes containing

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either the intact human IgG4 constant regions or the first constant region and hinge linker region from human IgG2 followed by the second and third constant regions from human IgG4 using standard molecular biology techniques. Chimeric antibodies were produced in 293-EBNA cells and purified by chromatography on protein A Sepharose as described (Evans *et al.*, 1995*a*). Each chimeric antibody preparation was greater than 95% pure as judged by Coomassie staining of SDS polyacrylamide gels. F(ab')2 was prepared by digestion of the antibodies with ficin (Pierce, Rockford, IL, U.S.A.) followed by protein A chromatography.

Flow cytometry

Cells used were obtained by trypsinization (PAEC and 293-EBNA) or centrifugation (Ramos, Jurkat, YT and U-937 cells). Harvested cells were washed once with 4°C PBS containing 1% heat-inactivated FBS (PBS/1) and aliquoted at 50 000–100 000 cells per well in 96-well V-bottom plates (Nunc). Cells were washed two additional times with PBS/1, and then incubated with PBS/1 containing 10 μg/ml primary Ab or PBS/1 alone for 30 min at 4°C. For some experiments the chimeric HuG4 or HuG2/G4 was centrifuged at $1 \times 10^5 g$ for 1 hr prior to addition to the cells. The cells were then washed twice with PBS/1 and resuspended in PBS/1 containing the appropriate FITC-conjugated secondary antibodies (1:200 dilution) for 30 min at 4°C. The cells were again washed twice with PBS/1 and resuspended in PBS. Cell fluorescence was determined using a Becton Dickinson FACSort.

Adhesion assays

On day 0 PAEC were plated in medium A at a density of 10 000 cells per well in a 96-well flat bottom plate. On day 1 the PAEC were refed medium A supplemented with 1 μg/ml LPS (Sigma, *E. coli* 0111:B4). On day 2, human cells were resuspended in the appropriate growth medium supplemented with 10 μM calcein AM (Molecular Probes, Eugene, OR, U.S.A.) and incubated at 37°C for 30 min. The cells were subsequently washed three times with growth medium and resuspended at 6×10^6 cells/ml in RPMI 1640 supplemented with 1% heat-inactivated FBS (RPMI/1). The PAEC were washed twice with RPMI/1 and refed 50 μl/well of RPMI/1 containing the indicated concentrations of antibodies. After adding calcein-labeled cells at a density of 3×10^5 cells/well (50 μl), the plate was centrifuged at 200*g* for 1 min and incubated at 37°C for 15 min. The nonadherent cells were removed by washing three to five times with RPMI/1. Adherent cells were lysed with HBSS containing 1% SDS and calcein fluorescence was measured with a Millipore CytoFluor 2350 using an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Background fluorescence was determined from wells containing PAEC that did not receive calcein-labeled cells. Samples were performed in duplicate in each experiment. Adherence was determined by comparing the residual fluorescence concentration in each well to the input fluorescence concentration. In each experiment the specific fluorescence signal obtained in the absence of competing antibody was defined as 100%.

For adhesion assays using purified soluble pVCAM (Mueller *et al.*, 1995), PolySorp 96-well microtiter plates (Nunc) were coated overnight at 4°C with 200 ng/well purified soluble pVCAM in 0.1 M carbonate buffer, pH 9.6. After blocking the plate with RPMI 1640 supplemented with 10 mg/ml BSA (RPMI/BSA), the plates were incubated with RPMI/BSA containing antibody for 1 hr at 37°C. Subsequently, 3×10^5 calcein-labeled U-937 cells in RPMI/BSA were added to each well. The plate was centrifuged upright for 1 min at 200*g* and incubated at 37°C for 15 min. The plate was then centrifuged inverted at 200*g* for 3 min. Adherent cells were lysed by the addition of HBSS containing 1% SDS and calcein fluorescence was measured with a Millipore CytoFluor 2350 using an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

RESULTS

Identification of porcine VCAM domains recognized by blocking mAbs

Previously, we have identified two murine mAbs (3F4 and 2A2) directed against porcine VCAM (pVCAM) which block VLA-4 dependent adhesion of peripheral blood human T cells to PAEC. These two mAbs are specific for pVCAM and do not recognize human VCAM (hVCAM). Here an initial analysis of the epitope specificity of the 2A2 and 3F4 mAbs was performed using fusion proteins consisting of various domains of pVCAM or hVCAM joined to the Fc region of human IgG1. The VCAM/Ig fusion proteins were produced in 293-EBNA cells and assayed for binding of 2A2 and 3F4 mAbs by ELISA (Fig. 1). Both the 2A2 and 3F4 mAbs bound efficiently to proteins containing the first three domains of pVCAM. The 2A2 mAb bound to fusion proteins containing pVCAM domain 1 such as pD1 + pD2 (porcine domain 1 plus porcine domain 2) and pD1 + hD2 (porcine domain 1 plus human domain 2), but did not bind to fusion proteins lacking pVCAM domain 1 such as pD2 + pD3, hD1 + pD2, or hD1 + hD2. Weak binding of the 2A2 mAb could also be detected to a fusion protein containing only porcine domain 1 (pD1) but not to fusion proteins containing only pD2 or pD3. Conversely, the 3F4 mAb bound to fusion proteins containing pVCAM domain 2, such as pD1 + pD2, pD2 + pD3, and hD1 + pD2, but did not bind to fusion proteins lacking pVCAM domain 2, such as pD1 + hD2 and hD1 + hD2. Binding of the 3F4 mAb could not be detected to fusion proteins containing only a single pVCAM domain. The weak binding of 2A2 and 3F4 to fusion proteins containing only a single VCAM domain is similar to previously characterized blocking antibodies specific for human VCAM and ICAM-1 which require the presence of at least two adhesion molecule domains for binding (Pepinsky *et al.*, 1992; Renz *et al.*, 1994). Together, these results suggested the 2A2 mAb specificity is defined by

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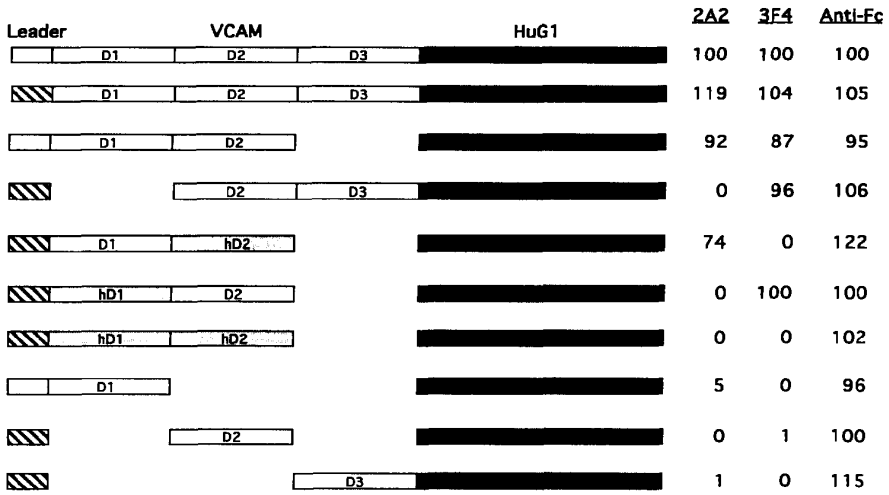


Fig. 1. Binding of 3F4 and 2A2 mAbs to VCAM/Ig fusion proteins. Proteins consisting of domains 1 (D1), 2 (D2), or 3 (D3) from porcine VCAM (open boxes) or human VCAM (gray boxes) joined to the Fc region of human IgG1 (black boxes) were produced in 293-EBNA cells. In some constructs the pVCAM secretory leader sequence was replaced with the secretory leader sequence from human CD59 (striped boxes). The fusion proteins were captured by incubating diluted conditioned media in 96-well plates coated with goat anti-human IgG (Fc specific). After incubation with 2A2 or 3F4 mAb, bound murine mAb was detected using peroxidase-conjugated goat anti-mouse IgG (Fc specific). The total amount of each fusion protein captured per well was quantitated using peroxidase-conjugated goat anti-human IgG (Fc specific). The absorbance signal obtained with the fusion protein containing the intact pVCAM domains 1, 2 and 3 was defined as 100% for each antibody. Binding of the 2A2 and 3F4 mAbs to the remaining fusion proteins is reported as a percentage of this value. Results shown are from a representative experiment of several performed.

pVCAM domain 1 whereas the 3F4 mAb specificity is defined by pVCAM domain 2.

Inhibition of human leukocyte binding to PAEC by chimeric anti-porcine VCAM mAbs

The use of murine antibodies in porcine-to-primate xenotransplantation models or ultimately in porcine-to-human xenotransplants poses several problems including a short half-life of the murine antibody plus development of an immune response directed against the murine antibody. These problems have been significantly reduced by the use of chimeric antibodies consisting of the murine variable regions joined to human constant regions (Khazaeli *et al.*, 1994). To create anti-pVCAM chimeric antibodies we cloned the variable regions from both 2A2 and 3F4. The murine variable heavy regions were joined to the human IgG4 constant region (HuG4) since this isotype does not activate complement (C) (Tao *et al.*, 1993), the predominant mediator of hyperacute rejection of xenografts. The murine variable light regions were joined to the human kappa constant region. Chimeric ch2A2 HuG4 and ch3F4 HuG4 antibodies were produced by coexpression of the chimeric heavy and light chains in 293-EBNA cells and tested for binding to pVCAM by FACS analysis using 293 cells expressing pVCAM (Fig. 2(A)). Both chimeric mAbs produced identical staining profiles as the corresponding murine mAbs, while neither chimeric mAb bound to untransfected 293 cells. Additionally, both the chimeric antibodies and murine mAbs produced identical FACS profiles using LPS-activated porcine aortic endothelial cells (Fig. 2(B)), confirming the chimeric antibodies retained the ability to bind to pVCAM expressed on PAEC.

The ability of the ch2A2 HuG4 and ch3F4 HuG4 antibodies to block the interaction between human VLA-4 and porcine VCAM was assessed by measuring inhibition of Ramos cell binding to activated PAEC (Fig. 3). The murine 3F4 mAb inhibited this binding by 90% when present at concentrations greater than 5 μg/ml. A control humanized antibody directed against human C5 (h5G1.1 HuG4) did not inhibit the binding of Ramos to activated PAEC. The ch2A2 HuG4 and ch3F4 HuG4 produced nearly identical inhibition curves as the murine 3F4 mAb, demonstrating the retention of binding avidity and cell adhesion blocking capabilities in these chimeric antibodies.

The ability of the ch2A2 HuG4 and ch3F4 HuG4 mAbs to inhibit binding of cells expressing both VLA-4 and LFA-1 was next examined using the Jurkat T cell line. Both the ch2A2 HuG4 and ch3F4 HuG4 antibodies inhibited the binding of Jurkat to activated PAEC by up to 90% while the control antibody h5G1.1 HuG4 did not inhibit binding at any concentration (Fig. 4(A)). Similarly, the ch2A2 HuG4 and ch3F4 HuG4 antibodies inhibited the binding of both resting (Fig. 4(B)) and activated (Fig. 4(C)) T cells to activated PAEC, although the

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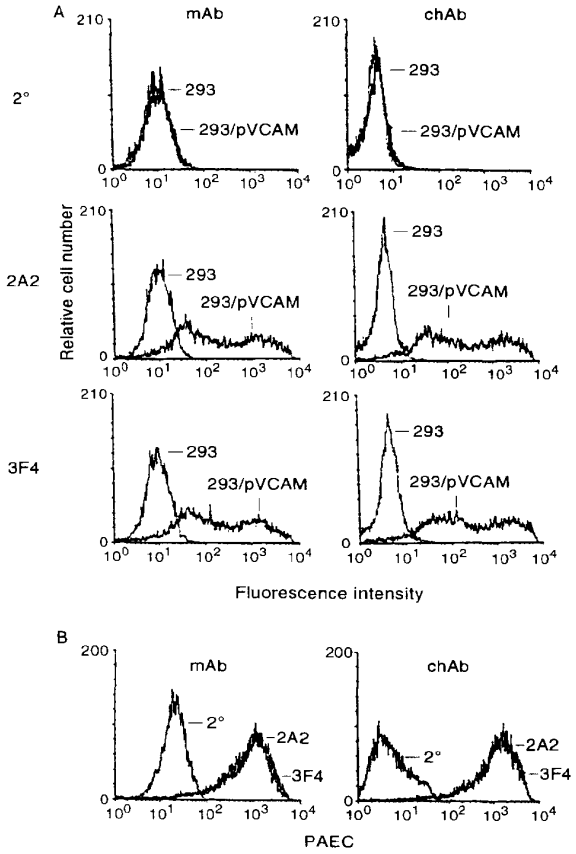


Fig. 2. Binding of 3F4 and 2A2 chimeric antibodies to pVCAM. (A) Chimeric mAbs containing the variable regions of either 2A2 or 3F4 joined to human IgG4 and kappa constant regions were produced in 293-EBNA cells and purified by protein G Sepharose chromatography. Untransfected 293-EBNA cells (293) or 293-EBNA cells expressing pVCAM (293/pVCAM) were incubated with 10 μg/ml purified murine 2A2 mAb, murine 3F4 mAb, ch2A2 HuG4, or ch3F4 HuG4. Bound antibody was detected using either FITC-conjugated goat anti-mouse IgG or FITC-conjugated goat anti-human IgG followed by flow cytometry using a Becton Dickinson FACSort. Data are displayed as histograms with fluorescence intensity plotted on the x-axis and relative cell number plotted on the y-axis. Background staining by the secondary antibodies is shown in the panels labeled 2°. (B) PAEC were activated by treatment with 1 μg/ml LPS for approximately 16 hr. Cells were then stained with either the murine 2A2 and 3F4 mAbs (left panel) or the ch2A2 HuG4 and ch3F4 HuG4 antibodies (right panel). Background staining by the secondary antibodies (2°) is indicated.

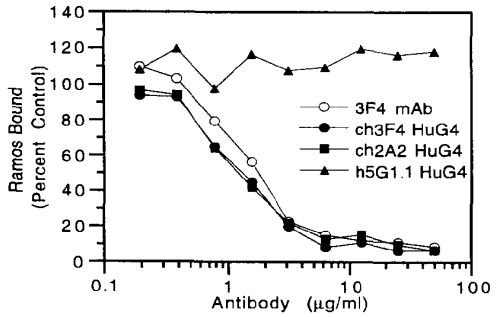


Fig. 3. Binding of Ramos cells to PAEC in the presence of mAbs. On day 0, PAEC were plated at 10 000 cells per well in medium A in 96-well plates. On day 1 the PAEC were activated by adding fresh medium A supplemented with 1 μg/ml LPS. After 16 to 24 hr incubation, the PAEC were washed and refed RPMI 1640 containing 1% FCS plus the indicated concentrations of the murine 3F4 mAb (3F4 mAb), the chimeric 2A2 and 3F4 mAbs (ch2A2 HuG4 and ch3F4 HuG4) or a humanized negative control antibody (h5G1.1 HuG4). Calcein-labeled Ramos cells were added and allowed to adhere to the PAEC under static conditions for 15 min. After nonadherent cells were removed by washing, the remaining bound cells were lysed with SDS. Calcein fluorescence of the SDS supernatant was measured with a Millipore CytoFluor 2350. In each experiment the binding of Ramos cells to PAEC in the absence of antibody was defined as 100%. Results shown are the mean of two to four experiments, with each value determined in duplicate in each experiment.

maximal inhibition achieved was reduced to approximately 60%. Addition of a blocking mAb directed against human VLA-4 also maximally inhibited adhesion by 60% (Fig. 4(C)), confirming the binding inhibition observed was due to blockade of the molecular interaction between human VLA-4 and porcine VCAM.

The potential of the ch2A2 HuG4 and ch3F4 HuG4 antibodies to inhibit binding of natural killer cells and monocytes to activated PAEC was examined using the cell lines YT (Yodio *et al.*, 1985) and U-937 (Sundstron and Nilsson, 1976), respectively. Binding of YT cells to activated PAEC was strongly inhibited by concentrations of 10 μg/ml ch2A2 HuG4 and ch3F4 HuG4 antibodies (Fig. 5(A)). In contrast, the ch2A2 HuG4 and ch3F4 HuG4 antibodies did not inhibit binding of U-937 cells to activated PAEC (Fig. 5(B)). One possible explanation for this failure to inhibit U-937 cell binding could be the presence of elevated levels of VLA-4 on U-937 cells. However, FACS analysis revealed the presence of comparable levels of CD49d and activated CD29 on U-937 cells as on other cell lines such as Jurkat whose adhesion to PAEC was inhibited by the chimeric anti-pVCAM antibodies (Fig. 6). Interestingly, the adhesion of U-937 to PAEC was consistently augmented by approximately 50% in all experiments. This increase was specific for the anti-pVCAM antibodies as no increase in U-937 binding to PAEC was detected in the presence of the control h5G1.1 HuG4 antibody (Fig. 5(B)). This suggested that binding of U-937 to PAEC could be increased through FcR binding to the anti-pVCAM HuG4 antibodies on the PAEC surface. In support of this hypothesis, FACS analysis confirmed the presence of significant levels of FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) on the surface of U-937 cells. In contrast, Jurkat and YT cells had no detectable Fcγ receptors while Ramos cells showed the presence of only low levels of FcγRII (Fig. 6).

Binding of the HuG4 chimeric antibodies to U-937 could be directly demonstrated by flow cytometry (Fig.

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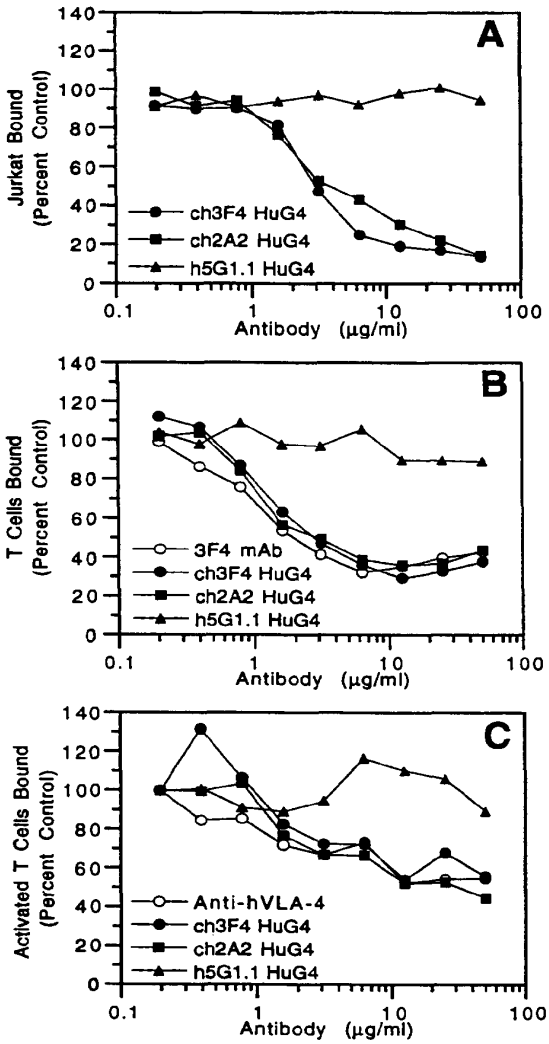


Fig. 4. Binding of Jurkat and human T cells to PAEC in the presence of HuG4 antibodies. Binding experiments were performed as described in Fig. 3 using calcein-labeled Jurkat cells (A) or calcein-labeled purified human T cells (B and C). Human T cells were either assayed immediately after purification (B) or cultured in the presence of 1 μg/ml PHA for 5 days followed by 1 day without PHA stimulation (C) prior to performing the adhesion assay. Results shown are the mean of two to four experiments (A and B) or from an individual experiment (C). Anti-hVLA-4 denotes the blocking murine mAb HP2/1 directed against the α4 subunit of human VLA-4.

7). Both the HuG4 antibodies as well as the anti-human C5 HuG4 antibody bound to U-937 cells. In contrast, 293/pVCAM cells bound only the anti-pVCAM antibodies but not the anti-human C5 HuG4 antibody. No binding of the HuG4 chimeric antibodies could be detected with Ramos, Jurkat, or YT cells. These results suggest that the HuG4 chimeric antibodies bound to U-937 cells through interaction of the HuG4 Fc region with the Fc receptors (FcRs) present on U-937 cells. Finally,

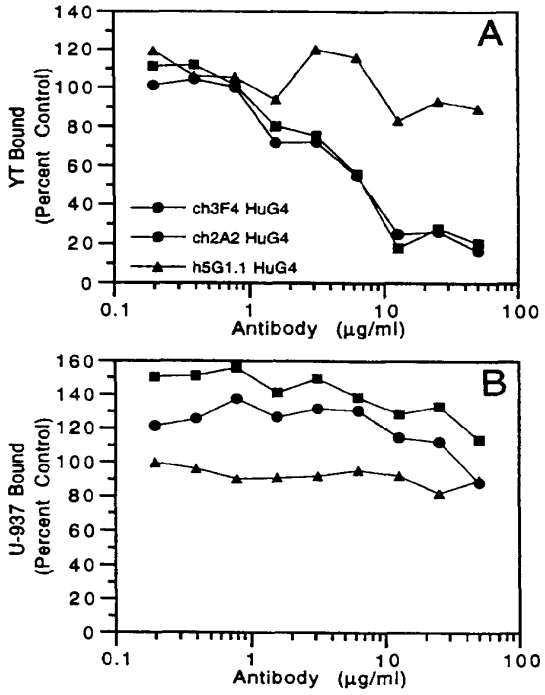


Fig. 5. Binding of YT and U-937 to PAEC in the presence of HuG4 mAbs. Adherence of calcein-labeled YT cells (A) or U-937 cells (B) to PAEC was assayed as described in Fig. 3. Results shown are the mean of two to four experiments.

F(ab')2 fragments prepared from ch3F4 HuG4 strongly inhibited binding of U-937 cells to PAEC (Fig. 8) whereas F(ab')2 fragments prepared from h5G1.1 HuG4 did not inhibit the binding. This result demonstrates that, if antibody crosslinking is prevented, blockade of the interaction between human VLA-4 and porcine VCAM can effectively inhibit the adhesion of human monocytes to PAEC.

Inhibition of FcR binding of humanized mAbs with altered constant regions

Although F(ab')2 fragments proved capable of inhibiting adhesion of U-937 cells, antibody fragments are suboptimal for chronic treatments, as they have much shorter *in vivo* half-lives than intact antibodies (Covell *et al.*, 1986). The use of intact antibodies with human constant regions as anti-rejection agents is complicated by the capacity of such antibodies to fix C and/or to bind FcRs. Human antibodies of the G4 isotype are devoid of C activity (Tao *et al.*, 1993; Smith *et al.*, 1995). Human antibodies of the G2 isotype do not activate C or bind FcRs (Canfield and Morrison, 1991; Burton and Woof, 1992). Rather than altering the specific residues known to mediate C fixation and FcR binding, we explored the possibility of creating an antibody with a composite human constant region. To create chimeric mAbs which would not activate C or bind to FcRs, we utilized the overall similarity in the human G2 and G4 primary

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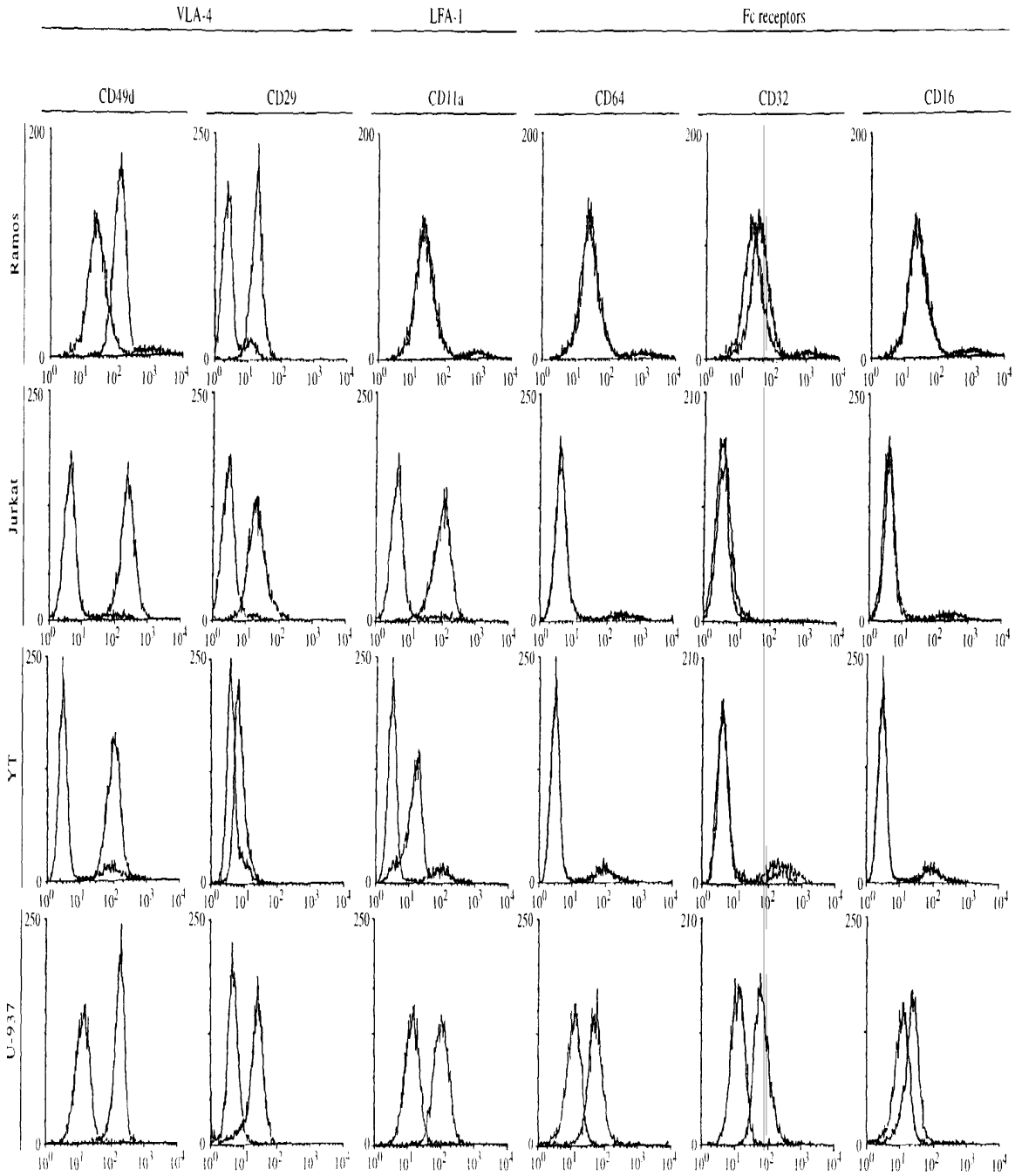


Fig. 6. Expression of VLA-4, LFA-1 and FcRs on the surface of Ramos, Jurkat, YT and U-937 cells. Cells were incubated with 10 μg/ml of the following primary antibodies: mouse anti-human CD49d, rat anti-activated human CD29, mouse anti-human CD11a, mouse anti-human CD64 ($Fc_\gamma RI$), mouse anti-human CD32 ($Fc_\gamma RII$) or mouse anti-human CD16 ($Fc_\gamma RIII$). After washing, cells were incubated with either FITC-conjugated goat anti-mouse IgG or FITC-conjugated goat anti-rat IgG as appropriate. Each panel presents the results obtained in the presence of primary plus secondary antibody or in the presence of secondary antibody alone.

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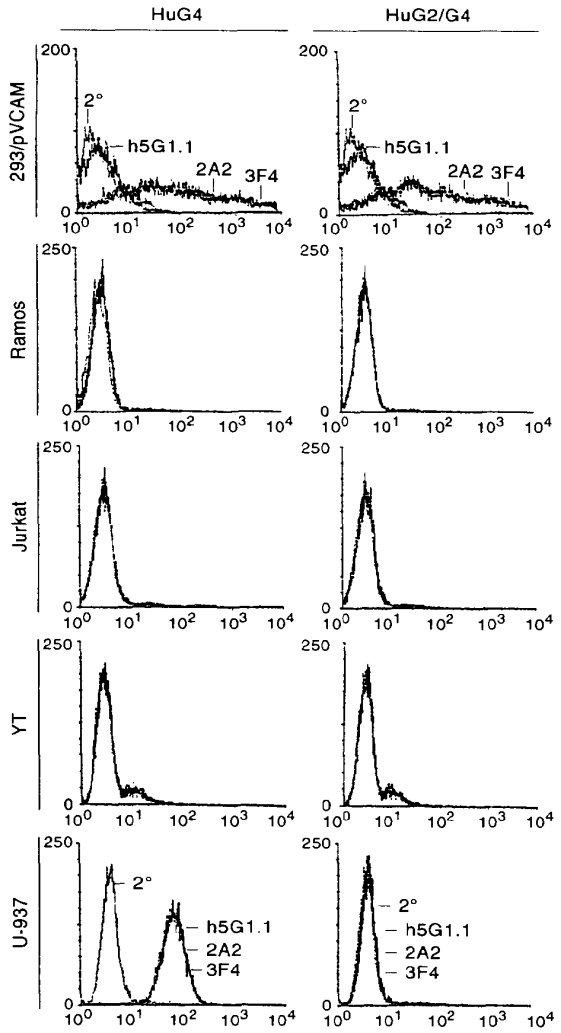


Fig. 7. Binding of chimeric antibodies to 293/pVCAM, Ramos, Jurkat, YT and U-937 cells. Cells were incubated with 10 μg/ml h5G1.1 HuG4, ch2A2 HuG4, or ch3F4 HuG4 (left panels) or 10 μg/ml h5G1.1 HuG2/G4, ch2A2 HuG2/G4, or ch3F4 HuG2/G4 (right panels). After washing, cells were incubated with FITC-conjugated goat anti-human IgG except for Ramos cells which were incubated with FITC-conjugated goat anti-human IgG (γ chain specific). Each panel presents the results obtained with the indicated cell line for h5G1.1 HuG4, ch2A2 HuG4 and ch3F4 HuG4 mAbs as well as the secondary antibody alone (2°) control.

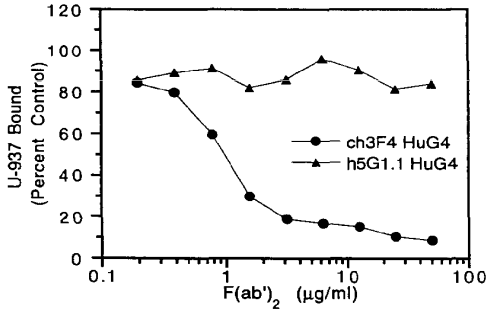


Fig. 8. Binding of U-937 cells to PAEC in the presence of F(ab')2 fragments. F(ab')2 fragments were prepared from h5G1.1 HuG4 and ch3F4 HuG4 by digestion with ficin. Undigested antibody and Fc fragments were removed by protein A chromatography. Adherence of calcein-labeled U-937 cells to PAEC in the presence of F(ab')2 was assayed as described in Fig. 3. Results shown are the mean of five experiments.

sequences to design a HuG2/G4 composite antibody consisting of the first constant region domain and the hinge region from the HuG2 isotype joined to the second and third constant region domains from the HuG4 isotype (Fig. 9). The resulting mAb did not contain the antibody sequences necessary for FcR binding, nor any new epitopes that would likely be immunogenic.

Binding to FcR was examined by using the monocytic cell line U937. FACS analysis demonstrated that the resulting ch3F4 HuG2/G4, ch2A2 HuG2/G4 and h5G1.1 HuG2/G4 antibodies did not bind to U-937 cells (Fig. 7). The ch3F4 HuG2/G4 and ch2A2 HuG2/G4 antibodies maintained the ability to bind pVCAM as demonstrated both by binding to 293/pVCAM cells (Fig. 7) and by inhibition of Jurkat and YT binding to activated PAEC (Fig. 10). For both Jurkat and YT cells inhibition of binding with the chimeric HuG2/G4 was equivalent to the HuG4 or original murine anti-pVCAM antibodies. The binding of U-937 cells to PAEC was now potently inhibited by the presence of either ch2A2 HuG2/G4 or ch3F4 HuG2/G4 antibodies. Finally, the chimeric antibodies were examined for the ability to block the adhesion of U-937 cells to purified soluble pVCAM (Fig. 11). Addition of the ch3F4 HuG4 antibody failed to inhibit binding of U-937 cells to pVCAM unless very high concentrations of antibody were added. In contrast the ch3F4 HuG2/G4 antibody potently inhibited the binding of U-937 cells to pVCAM, while the negative control h5G1.1 HuG2/G4 antibody did not block binding. Together, these results demonstrate the anti-pVCAM HuG2/G4 antibodies specifically inhibited the adhesive interaction of human VLA-4 with porcine VCAM and that disruption of this interaction was sufficient to inhibit binding of numerous human cell types to porcine endothelial cells.

DISCUSSION

Current success in allotransplantation has resulted from the development of effective global immunosuppressive therapies that prevent cellular graft rejection. Recent attempts to develop more specific immunosuppressive strategies have focused on inhibiting interactions between integrins and costimulatory molecules on T cells with their ligands on graft endothelial cells and other antigen presenting cells (reviewed in Faustman, 1995; Guinan *et al.*, 1994). The success of allotransplantation has been limited by the resulting shortage of human donor organs. Recent progress in overcoming the initial

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HuG2      STKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGV
HuG2/G4   STKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGV
HuG4      STKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGV

HuG2      HTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVE
HuG2/G4   HTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVE
HuG4      HTFPAVLQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKVDKRVE

                                235
HuG2      RKCCVECPPCPAPPV- AGPSVFLF
HuG2/G4   RKCCVECPPCPAPPV- AGPSVFLF
HuG4      SKYGPPCPSCPAPEFLGGPSVFLF

HuG2      PPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPR
HuG2/G4   PPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPR
HuG4      PPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPR

                                                      331
HuG2      EEQFNSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTKG
HuG2/G4   EEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKG
HuG4      EEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKG
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Fig. 9. Sequence of the CH1, hinge and CH2 regions of human IgG2 (HuG2), human IgG4 (HuG4), and the chimeric HuG2/G4 construct. Positions where differences occur between the three molecules are double underlined. The amino acid sequence at position 235/236 determines ability to bind to Fcγ receptors (Canfield and Morrison, 1991; Duncan *et al.*, 1988; Alegre *et al.*, 1992; Burton and Woof, 1992) while the presence of a serine at position 331 confers the inability to activate C (Tao *et al.*, 1993).

hyperacute rejection of discordant xenografts suggests the potential for use of porcine xenografts as a clinical option to circumvent this chronic shortage. Although there are limited data regarding the fate of discordant xenografts which survive the initial hyperacute rejection phase, xenograft recipients will likely require continued cellular immunosuppression for the prevention of graft rejection. In this light, xenografts offer a unique opportunity for the development of blocking reagents specific for the graft, thereby avoiding global immunosuppression of the recipient.

To investigate the interactions occurring between human leukocytes and porcine endothelial cells, we have cloned the porcine VCAM molecule and created mAbs specific for porcine VCAM that do not bind to human VCAM (Mueller *et al.*, 1995). Two of these antibodies (3F4 and 2A2) are capable of blocking the interaction between human VLA-4 and porcine VCAM. Here we demonstrate that the binding specificity for 2A2 is located within domain 1 of pVCAM while the binding specificity for 3F4 is located within domain 2. This is identical to the results found with human VCAM, where blocking antibodies have also been shown to be directed against either domains 1 or 2 (Osborn *et al.*, 1992; Vonderheide and Springer, 1992).

The use of murine mAbs in humans and primates presents numerous problems including a short antibody half-life and a high degree of immunogenicity (Khazaeli *et al.*, 1994). To optimize the use of the anti-porcine VCAM antibodies in porcine-to-primate xenograft experiments we developed chimeric antibodies using the variable regions cloned from the 2A2 and 3F4 antibodies joined to the constant regions of the human IgG4,κ isotype. Given the extensive activation of C which occurs within xenografts, the human IgG4 isotype was chosen due to its inability to activate C. The resultant chimeric antibodies (ch2A2 HuG4 and ch3F4 HuG4 antibodies) retained specificity for porcine VCAM and were as potent as the original murine mAbs at blocking adhesion of Ramos cells to activated PAEC by greater than 90% (Fig. 3). In contrast to Ramos, the binding of resting or activated human T cells to activated PAEC was inhibited only approximately 50% by either the chimeric antibodies or the original mAbs (Fig. 4). Activation of human T cells by PAEC can be blocked by antibodies directed against either CD2 or LFA-1 (Rollins *et al.*, 1994*b*), suggesting either of these molecules could mediate the remaining adhesion by interacting with porcine CD58 or porcine ICAM-1, respectively.

The adhesion of human lymphocytes, monocytes or NK cells to activated HUVEC is dependent on the interaction of both LFA-1 with ICAM-1 and VLA-4 with VCAM (Allavena *et al.*, 1991; Jonjic *et al.*, 1992; Vonderheide and Springer, 1992). For each cell type, blocking the VLA-4/VCAM interaction by itself produces only a small reduction in adhesion, while antibodies which disrupt the LFA-1/ICAM-1 interaction provide a greater, although still partial, inhibition of adhesion. Addition of antibodies blocking both VLA-4/VCAM and LFA-1/ICAM-1 is required to completely block adhesion of these cell types to HUVEC. Surprisingly, binding of LFA-1$^+$–VLA$^+$4 Jurkat or YT cells to activated PAEC could be inhibited nearly completely by the ch3F4 HuG4 and ch2A2 HuG4 antibodies. One possible explanation for this could be the lack of interaction between human LFA-1 and porcine ICAM. The conservation of ICAM-1 is relatively low between species, with human and mouse ICAM-1 being only 50% conserved at the amino acid level (reviewed in Carlos and Harlan, 1994), and *in vitro* mouse LFA-1 does not bind to human ICAM-1. Alternatively, human LFA-1 may interact with porcine ICAM-1, but with a reduced affinity. For example, human VLA-4 binds to human VCAM at two independent sites, one within domain 1 and one within

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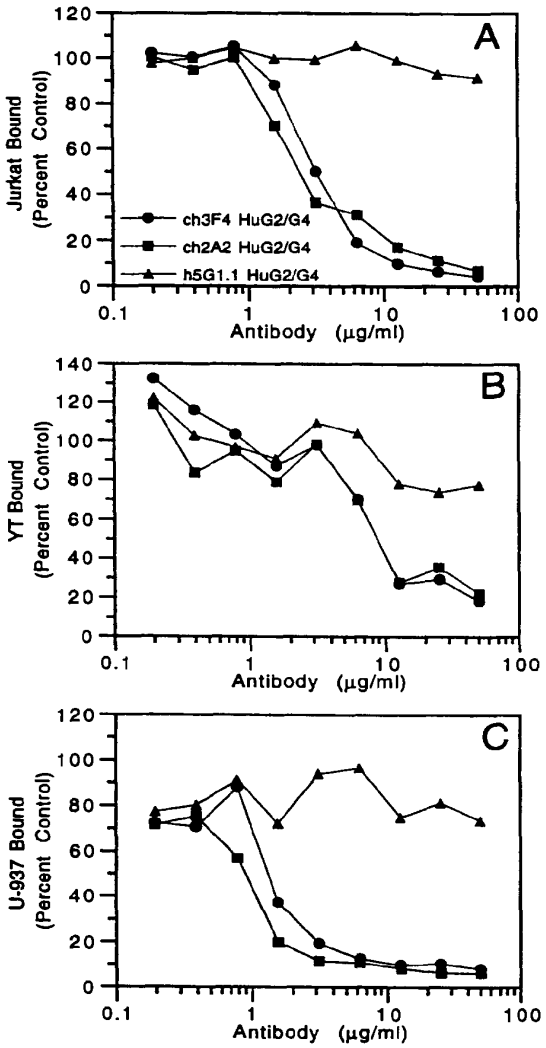


Fig. 10. Binding of Jurkat, YT and U-937 to PAEC in the presence of HuG2/G4 mAbs. Adherence of calcein-labeled Jurkat (A), YT (B) and U-937 (C) cells to PAEC was assayed as described in Fig. 3. Results shown are the mean of two to four experiments.

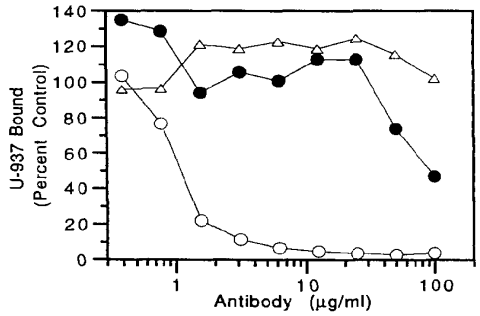


Fig. 11. Binding of U-937 to purified pVCAM. Microtiter plate wells were coated with purified soluble pVCAM. After blocking with FCS, wells received the indicated concentrations of either ch3F4 HuG4 (●), ch3F4 HuG2/G4 (○) or h5G1.1 HuG2/G4 (△) followed by 3×10^5 calcein-labeled U-937 cells. The cells were allowed to adhere at 37°C for 15 min after which non-adherent cells were removed by washing. Adherence of U-937 cells in the absence of competitor antibody was defined as 100%. Results shown are from an individual representative experiment.

domain 4 (Osborn *et al.*, 1992; Vonderheide *et al.*, 1994). In contrast, porcine VCAM lacks domain 4, suggesting the interaction between human VLA-4 and porcine VCAM may be weaker than the interaction between human VLA-4 and human VCAM. Reduced ligand affinities could make the binding of human effector cells to porcine endothelial cells more susceptible to disruption of any one of multiple weak interactions. These observations suggest the potential for significant differences in the therapeutic efficacy achieved by inhibiting particular receptor–ligand interactions with xenografts as compared to allografts.

Although the ch3F4 HuG4 and ch2A2HuG4 blocked binding of Jurkat and YT to activated PAEC, both antibodies failed to block binding of the monocytic U-937 cells to activated PAEC (Fig. 5). The levels of activated $\beta 1$ were comparable on Jurkat and U937 cells indicating an increased level of functional VLA-4 was not the mechanism behind the lack of inhibition of U-937 binding. Additionally, flow cytometry demonstrated the direct binding of the chimeric HuG4 antibodies to U-937 cells (Fig. 7). This binding was not dependent on the antigen specificity of the variable region, suggesting the interaction occurred through the Fc portion of the antibody. Finally, F(ab′)2 prepared from 3F4 HuG4 were able to block the adhesion of U-937 cells to activated PAEC by greater than 80% (Fig. 8). Together these results were consistent with a model in which the chimeric anti-pVCAM HuG4 mAbs crosslinked U-937 cells to activated PAEC by simultaneously binding FcRs and cell surface VCAM present on the PAEC. This is comparable to the situation encountered with the anti-CD3 murine monoclonal antibody (mAb) OKT3, which when first administered to patients produces crosslinking of T cells to FcR bearing cells, resulting in the activation of both cell types (Ceuppens *et al.*, 1985; Kan *et al.*, 1986) with resultant toxicity to the patient.

The interaction of antibodies with Fcγ receptors has been found to be dependent on the sequence of the hinge linker region of the CH2 domain (Duncan *et al.*, 1988; Alegre *et al.*, 1992; Burton and Woof, 1992) as well as an additional bend located between two β strands within the CH2 domain (Canfield and Morrison, 1991). In the case of a humanized OKT3 antibody (human IgG4 constant regions), binding to Fcγ receptors has been eliminated by the introduction of amino acid mutations in the hinge linker (Alegre *et al.*, 1992, 1994). In either case the resulting human IgG4 constant region contains amino acid differences which could be immunogenic. Here, we have created chimeric antibodies (designated HuG2/G4) containing the CH1 domain and hinge linker region of

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human IgG2 followed by the CH2 and CH3 domains of the human IgG4 constant region. Due to the similarities between these two isotypes at the amino acid level, the resultant antibody contained no novel peptides less than 30 amino acids in length likely to be presented by MHC class II molecules and invoke an immune response. The chimeric HuG2/G4 antibodies no longer bound to U-937 cells. Moreover, the ch2A2 HuG2/G4 and ch3F4 HuG2/G4 antibodies blocked the binding of Jurkat and YT cells to activated PAEC as potently as the chimeric HuG4 antibodies, demonstrating the alteration of the Fc region did not reduce the antibody avidities. Moreover, the chimeric anti-pVCAM HuG2/G4 recombinant antibodies nearly completely blocked the binding of U-937 cells to activated PAEC and to purified pVCAM. Finally, as predicted from the low affinity of IgG2 and IgG4 for human C1q, our preliminary results indicate that the HuG4 and HuG2/G4 recombinant antibodies show little or no C1q binding (data not shown).

In summary, we have developed humanized mAbs specific for porcine VCAM which potently block the adhesion of multiple human inflammatory cell types to porcine endothelial cells. The lack of binding of these antibodies to human VCAM leaves intact the interaction of human leukocytes with human endothelial cells. The adhesion-blocking properties of these antibodies will allow delineation of the role of VLA-4/VCAM interactions in cellular mediated rejection of porcine-to-primate xenografts. Additionally, the HuG2/G4 antibody design should prove useful in humanization of other antibodies intended for human use where elimination of FcR binding and C activation may be desirable.

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Sustained response and long-term safety of eculizumab in paroxysmal nocturnal hemoglobinuria

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Paroxysmal nocturnal hemoglobinuria (PNH) is a hematologic disorder characterized by clonal expansion of red blood cells (RBCs) lacking the ability to inhibit complement-mediated hemolysis. Eculizumab, a humanized monoclonal antibody that binds the C5 complement protein, blocks serum hemolytic activity. This study evaluated the long-term safety and efficacy of eculizumab in 11 patients with PNH during an open-label extension trial. After completion of an initial 12-week study, all patients chose to participate in the 52-week extension study. Eculizumab, administered at 900 mg every 12 to 14 days, was sufficient to completely and consistently block complement activity in all patients. A dramatic reduction in hemolysis was maintained throughout the study, with a decrease in lactate dehydrogenase (LDH) levels from 3110.7 IU/L before treatment to 622.4 IU/L ($P = .002$). The proportion of PNH type III RBCs increased from 36.7% at baseline to 58.4% ($P = .005$). The paroxysm rate of days with gross evidence of hemoglobinuria per patient each month decreased from 3.0 during screening to 0.2 ($P < .001$) during treatment. The median transfusion rate decreased from 1.8 U per patient each month before eculizumab treatment to 0.3 U per patient each month ($P = .001$) during treatment. Statistically significant improvements in quality-of-life measures were also maintained during the extension study. Eculizumab continued to be safe and well tolerated, and all patients completed the study. The close relationship between sustained terminal complement inhibition, hemolysis, and symptoms was demonstrated. (Blood. 2005; 106:2559-2565)

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Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a hematopoietic stem-cell disorder characterized by red blood cell (RBC) destruction, anemia, hemoglobinuria, and thrombosis. The intravascular hemolysis in PNH is continuous, with episodes of dark urine, or paroxysms, occurring at times of particularly brisk hemolysis. Ongoing hemolysis and/or insufficient hematopoiesis often result in transfusion dependence. Hemolysis in patients with PNH can be monitored by levels of the enzyme lactate dehydrogenase (LDH), which is typically elevated and can exceed 20 times the upper limit of normal during severe paroxysms.[1-3] There is no effective treatment for the ongoing hemolysis in PNH.

PNH results from the clonal expansion of somatically mutated hematopoietic stem cells. The predominant mutation results in a functional deficiency in phosphatidylinositol glycan class A (PIG-A), a protein that is critical for the biosynthesis of the glycosylphosphatidylinositol (GPI) anchor, a mechanism by which various proteins are attached to the cell membrane.[4,5] Consequently, there is a partial (type II) or complete (type III) deficiency of GPI-anchored proteins on the surfaces of PNH hematopoietic stem cells and their progeny. Two such proteins are the complement inhibitors CD55 and CD59. CD55 inhibits complement at the level of C3, whereas CD59 prevents terminal complement components from forming the hemolytic membrane pore (C5b-9).[6-8] Deficiency of these complement inhibitors renders PNH RBCs sensitive to complement-mediated lysis.[7]

Eculizumab is a humanized monoclonal antibody that specifically targets the complement protein C5 and prevents its cleavage.[9] C5 is the point at which the pathways of complement activation converge, and it is the first protein of terminal complement assembly. Complement inhibition at this stage blocks the generation of C5a and the formation of C5b-9 while it preserves early complement components that are critical for the clearance of microorganisms and immune complexes.[10]

We previously reported the outcome of an open-label study of eculizumab in patients with PNH.[2] Results of this 12-week study demonstrated a dramatic reduction in hemolysis and a concomitant increase in the proportion of PNH type III RBCs. In addition, this initial study showed a marked decrease in the rates of paroxysms and blood transfusions and an improvement in quality of life. Here we report the results of a 1-year follow-up study designed to assess the long-term efficacy and safety of eculizumab in patients with PNH.

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An Inside *Blood* analysis of this article appears at the front of this issue.

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Patients, materials, and methods

Trial design

The acute-phase study was an initial 12-week, open-label trial of eculizumab in 11 patients with PNH and has been described previously in detail.[2] The current study was an open-label extension of that acute-phase study. All 11 patients from the acute-phase study enrolled in the extension study. Patients were allowed concomitant therapy—with the exception of whole blood, which contains C5—at the discretion of their treating physicians. Two of 11 patients had a history of thrombosis before eculizumab treatment, and 6 of 11 patients were on warfarin therapy before and during the trial.

The trial was approved by the Leeds (West) Research Ethics Committee, United Kingdom, and was performed according to the International Conference on Harmonisation and Good Clinical Practice Standards. All patients gave written informed consent and were treated with eculizumab.

Eculizumab administration

All patients entered the extension study on a maintenance dose of eculizumab (at the conclusion of the acute-phase study). This maintenance dose of 900 mg intravenously every 14 days was continued throughout the extension study period. Two patients, however, required that the dosing interval be shortened to every 12 days so that consistent and complete complement inhibition could be maintained.

Clinical investigations

As in the acute-phase study, data were obtained in the open-label extension study on the pharmacokinetics (PK), pharmacodynamics (PD), and immunogenicity of eculizumab, indicators of hemolysis, PNH clone size, paroxysm and transfusion rates, and quality-of-life measurements. The trigger for transfusion before and during the study remained unchanged for each patient and was based on a combination of hemoglobin levels and the occurrence of symptoms resulting from anemia, hemolysis, or both. This information has been described in detail elsewhere.[2]

Statistical analysis

Biochemical values were compared using the paired Student t test, change of transfusion and paroxysm rates were analyzed using the Wilcoxon signed rank test, quality-of-life measurements were analyzed using mixed-effect analysis of variance, and comparison of the number of days with paroxysms was analyzed using the Fisher exact test.

Results

Pharmacokinetics and pharmacodynamics

In the acute-phase study, 10 of 11 patients maintained sufficient levels of eculizumab (≥ 35 μg/mL) for terminal complement to be sufficiently inhibited (≤ 20% serum hemolytic activity) for the duration of the 12-week treatment period (Figure 1, weeks 0-12). During the extension study, 9 of 11 patients continued to show complete complement blockade throughout the 52-week treatment period (Figure 1, weeks 12-64). The 2 patients whose serum eculizumab levels decreased below 35 μg/mL (Figure 1A, lines 1-2) showed a return of serum hemolytic activity (Figure 1B, lines

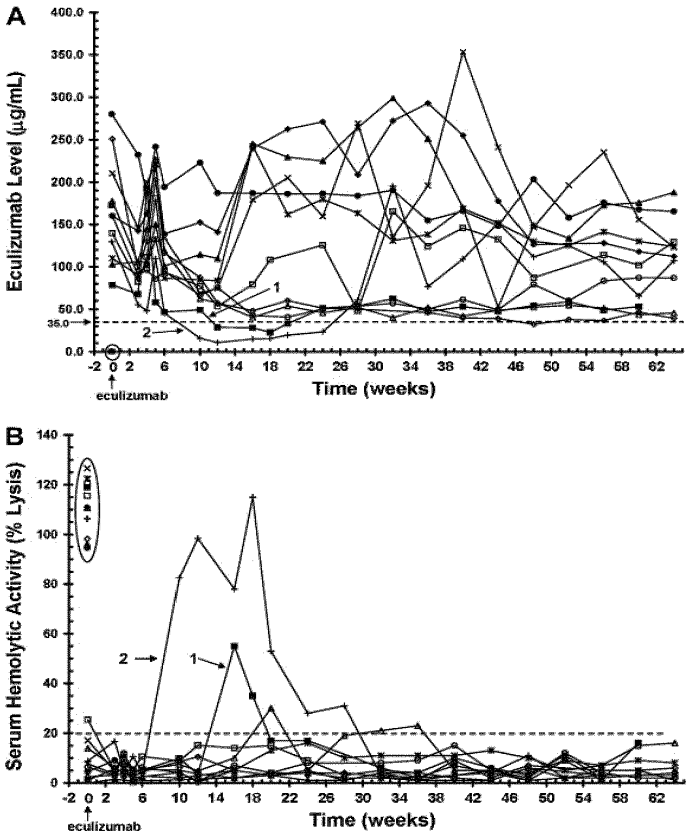


Figure 1. PK and PD analyses of eculizumab in patients with PNH. Initiation of eculizumab dosing is indicated at time 0 on the x axis. (A) Serum levels of eculizumab in 11 patients with PNH during the 64 weeks of treatment. The dashed line indicates the level of eculizumab required to completely block complement activity (≥ 35 μg/mL). Time 0 shows levels of eculizumab before (encircled) and 1 hour after dosing, whereas all other time points represent trough values. Two patients with trough levels of eculizumab below 35 μg/mL during the maintenance dosing are identified (patients 1 and 2). (B) Serum hemolytic activity (PD) during the 64-week treatment period, as determined by the ability of serum to lyse antibody-presensitized chicken erythrocytes. The dashed line indicates the percentage of hemolytic activity at which complement is considered effectively inhibited (≤ 20%). Time 0 shows serum hemolytic activity before (encircled) and 1 hour after dosing, whereas all other time points represent trough values. Two patients with trough serum hemolytic activity values above 20% are identified (patients 1 and 2).

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1-2). However, a reduction in the dosing interval from 900 mg every 14 days to 900 mg every 12 days (initiated between weeks 18 and 24) in the 2 breakthrough patients was adequate to keep trough levels of eculizumab higher than 35 μg/mL (Figure 1A), thereby completely blocking serum complement activity for the remainder of the extension study (Figure 1B).

Measures of hemolysis

Lactate dehydrogenase (LDH) is a standard biochemical measure of intravascular hemolysis, and levels are frequently elevated in patients with PNH.[1-3] The immediate reduction in LDH levels observed in all patients during the acute-phase study was maintained during the extension study (Figure 2). Two patients demonstrated transient increases in LDH levels (Figure 2, lines 1-2) that correlated temporally with the breakthroughs in serum complement activity shown in Figure 1. As complement blockade was restored, LDH levels returned to near-normal values.

Levels of various markers of hemolysis and platelet counts during eculizumab treatment are shown in Table 1. LDH levels decreased from a mean of 3110.7 ± 598.4 IU/L during the 52 weeks before treatment to 594.0 ± 31.7 IU/L and 622.4 ± 41.1 IU/L (normal range at Leeds Teaching Hospitals, 150-480 IU/L) during the 12 and 64 weeks of treatment, respectively ($P = .002$ for 64-week comparison in all patients). Similarly, aspartate aminotransferase (AST) levels, another marker of hemolysis, decreased from a mean baseline value of 76.2 ± 16.0 IU/L to 26.2 ± 2.3 IU/L and 30.1 ± 3.2 IU/L (normal range at Leeds Teaching Hospitals, 10-40 IU/L) during the 12 and 64 weeks of treatment, respectively ($P = .02$ for 64-week comparison in all patients). The dramatic reduction in hemolysis during eculizumab treatment was demonstrated in both noncytopenic (platelet count $\geq 150 \times 10^9$/L) and cytopenic (platelet count $< 150 \times 10^9$/L) patient populations. Levels of haptoglobin, hemoglobin, and bilirubin and numbers of reticulocytes and platelets did not change significantly in the comparison of prestudy values and 64-week treatment values.

Proportions of PNH blood cells

Clonal expansion of hematopoietic stem cells with a reduction in or an absence of GPI-linked membrane proteins (type II or type III cells, respectively) is a hallmark of PNH. The effect of eculizumab on the proportions of various PNH blood-cell types was assessed (Table 2). The proportion of PNH type III RBCs relative to the total RBC population increased from 36.7% ± 5.9% at baseline to 59.2% ± 8.0% and 58.4% ± 8.5% during the 12 and 64 weeks of treatment, respectively ($P = .005$ for 64-week comparison in all patients). The increase in the proportion of PNH type III RBCs was more pronounced in patients without cytopenia than in those with cytopenia. However, the lower proportion of type III RBCs in patients with cytopenia was a direct result of the dilutional effect from the increased administration of normal RBCs because of the higher transfusion requirement in this patient group. The proportion of PNH type II RBCs increased from 5.3% ± 1.4% before treatment to 13.2% ± 2.4% ($P = .013$) during the 64 weeks of treatment. Mean proportions of type III neutrophils and platelets were greater than 90% before eculizumab therapy and were stable during the study.

Paroxysm and transfusion rates

The presence of hemoglobin in the urine (hemoglobinuria) is characteristic of PNH and a central component of periodic exacerbations known as paroxysms. In the eculizumab study, paroxysm rates were defined as the number of days with the presence of gross hemoglobinuria, defined as a colorimetric urine score of 6 or greater on a scale of 1 to 10.[2] In patients whose urine scores were assessed, the paroxysm rate decreased from 3.0 paroxysms per patient each month before eculizumab treatment to 0.1 paroxysms per patient each month during the initial 12 weeks and 0.2 paroxysms per patient each month during the total 64 weeks of treatment (Figure 3; $P < .001$).

During the extension study, 2 patients did not sustain levels of eculizumab necessary to consistently block complement (Figure 1). 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. A detailed analysis of the dosing interval between visits 4 and 5 in one of the breakthrough patients showed that sufficient levels of eculizumab (PK ≥ 35 μg/mL) were present to completely block serum hemolytic activity (PD ≤ 20% hemolytic activity) for the first 12 days (Figure 4). Effective complement blockade during these 12 days was reflected by normal urine color scores and low LDH and AST levels. On the 13th and 14th days of the dosing interval, a paroxysm occurred, as evidenced by severe hemoglobinuria (black urine), dysphagia, and dramatic increases in LDH and AST levels. These events correlated with insufficient levels of eculizumab (PK) and the return of serum hemolytic activity (PD). The patient was again dosed on day 14, which resulted in the resolution of hemoglobinuria and dysphagia by the next morning. A reduction in the dosing interval from 14 to 12 days between visits 5 and 9 was sufficient to maintain the levels of

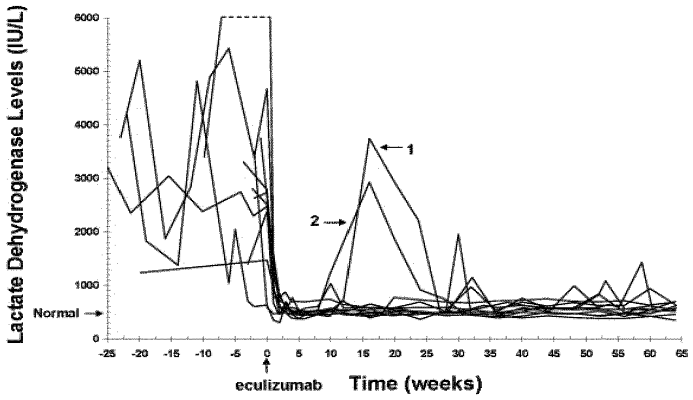


Figure 2. LDH levels in patients with PNH before and during eculizumab treatment. Initiation of eculizumab dosing is indicated at time 0 on the x axis. LDH values are shown for 11 patients with PNH for 25 weeks before and 64 weeks during eculizumab treatment. (Normal) Upper limit of normal of the LDH range at the Leeds Teaching Hospital. The dashed line indicates off-scale points from one patient with a peak value of 12 100 IU/L. Two patients who experienced a return of serum hemolytic activity during treatment are identified (patients 1 and 2).

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Table 1. Changes in levels of various markers of hemolysis and platelet counts during eculizumab therapy

Biochemical marker	Normal range	Time of analysis‖			P*
		Before study†	12 wk	64 wk	
LDH, IU/L					
All patients	150-480	3110.7 ± 598.4	594.0 ± 31.7	622.4 ± 41.1	.002
Patients without cytopenia‡	NA	3965.0 ± 971.7	617.2 ± 24.3	657.1 ± 54.8	—
Patients with cytopenia§	NA	2085.0 ± 267.0	566.3 ± 65.5	580.8 ± 63.2	—
AST, IU/L					
All patients	10-40	76.2 ± 16.0	26.2 ± 2.3	30.1 ± 3.2	.02
Patients without cytopenia	NA	88.5 ± 28.3	24.4 ± 3.8	30.8 ± 5.7	—
Patients with cytopenia	NA	61.4 ± 9.8	28.4 ± 2.4	29.1 ± 2.3	—
Haptoglobin, g/L					
All patients	0.5-2	0.06 ± 0	0.07 ± .01	0.14 ± 0.07	NS
Patients without cytopenia	NA	0.06 ± 0	0.08 ± .02	0.20 ± 0.12	—
Patients with cytopenia	NA	0.06 ± 0	0.06 ± 0	0.08 ± 0.01	—
Hemoglobin, g/dL					
All patients	11.5-16	10.0 ± 0.4	10.3 ± 0.4	10.4 ± 0.4	NS
Patients without cytopenia	NA	10.4 ± 0.5	10.9 ± 0.6	10.8 ± 0.5	—
Patients with cytopenia	NA	9.5 ± 0.7	9.6 ± 0.4	9.9 ± 0.5	—
Bilirubin, μM					
All patients	3-15	25.9 ± 4.3	28.2 ± 4.4	28.7 ± 4.0	NS
Patients without cytopenia	NA	30.6 ± 7.4	34.9 ± 6.6	34.6 ± 5.9	—
Patients with cytopenia	NA	20.2 ± 2.2	20.2 ± 3.6	21.7 ± 3.6	—
Reticulocytes, $\times 10^{-3}/mm^3$					
All patients	20-80	161.4 ± 25.9	191.2 ± 23.6	189.6 ± 21.8	NS
Patients without cytopenia	NA	200.2 ± 38.4	243.4 ± 26.2	233.2 ± 27.4	—
Patients with cytopenia	NA	114.8 ± 21.9	128.5 ± 15.6	137.3 ± 15.2	—
Platelets, $\times 10^9/L$					
All patients	150-400	183.0 ± 35.3	183.6 ± 37.8	180.8 ± 35.8	NS
Patients without cytopenia	NA	250.2 ± 49.8	256.3 ± 51.8	251.5 ± 47.2	—
Patients with cytopenia	NA	102.2 ± 11.8	96.3 ± 18.7	95.9 ± 19.7	—

— indicates not determined; NA, not applicable; and NS, not significant.
*Comparisons of mean change from the prestudy period to the 64-week treatment period for all patients.
†Mean values during 52-week period before treatment except for AST, which represents the baseline mean.
‡Platelet count ≥ 150×10^9/L; n = 6.
§Platelet count < 150×10^9/L; n = 5.
‖Values are presented as ± standard error (SE).

Table 2. Changes in proportions of PNH blood-cell types during eculizumab treatment

PNH cell type	Proportion of PNH cells, %			P*
	Baseline§	12 wk§	64 wk§	
Type III RBCs				
All patients	36.7 ± 5.9	59.2 ± 8.0	58.4 ± 8.5	.005
Patients without cytopenia†	38.8 ± 3.7	73.5 ± 4.1	67.8 ± 7.0	—
Patients with cytopenia‡	34.2 ± 12.9	42.1 ± 13.8	47.0 ± 16.3	—
Type II RBCs				
All patients	5.3 ± 1.4	7.5 ± 2.1	13.2 ± 2.4	.013
Patients without cytopenia	4.8 ± 1.6	6.8 ± 3.0	12.9 ± 1.9	—
Patients with cytopenia	5.8 ± 2.4	8.4 ± 3.2	13.5 ± 5.0	—
Type III WBCs				
All patients	92.1 ± 4.6	89.9 ± 6.6	91.1 ± 5.8	NS
Patients without cytopenia	95.1 ± 2.3	94.9 ± 2.6	96.1 ± 1.1	—
Patients with cytopenia	88.4 ± 10.2	83.9 ± 14.5	85.2 ± 12.9	—
Type III platelets				
All patients	92.4 ± 2.4	93.3 ± 2.8	92.8 ± 2.6	NS
Patients without cytopenia	93.2 ± 1.4	95.8 ± 2.1	95.0 ± 0.8	—
Patients with cytopenia	91.6 ± 5.4	90.2 ± 5.6	90.2 ± 5.8	—

— indicates not determined; WBCs, white blood cells; and NS, not significant.
*Comparisons of mean change from baseline to the 64-week treatment period for all patients.
†Platelet count ≥ 150×10^9/L; n = 6.
‡Platelet count < less than 150×10^9/L; n = 5.
§Data are presented as ± SE.

eculizumab necessary to effectively and consistently block serum hemolytic activity and, therefore, intravascular hemolysis in these patients (Figure 4, visit 9).

A statistically significant reduction in the rate of packed red blood cell (PBRC) transfusion was observed in the acute-phase study and was maintained during the extension study compared with the rate of transfusion before eculizumab therapy (Table 3). Mean transfusion rates decreased from 2.1 U per patient each month during the 1-year period before treatment to 0.6 U per patient each month during the initial 12-week and to 0.5 U per patient each month during the total 64-week treatment period.

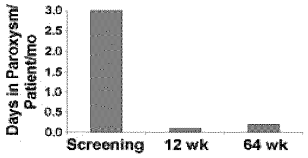
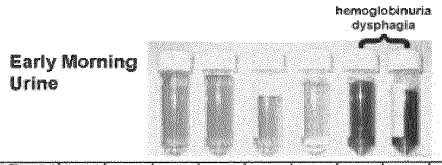


Figure 3. Paroxysm rate in patients with PNH before and during eculizumab treatment. A urine color scale[2] was used to assess the incidence of paroxysms in 8 patients with PNH before and during treatment with eculizumab. Paroxysm was prospectively defined by a urine colorimetric score of 6 or more. Bars represent the paroxysm rates (number of paroxysms per patient per month) during the screening period, during the first 12 weeks, and over the total 64 weeks of eculizumab treatment. Three patients were not included in the analysis either because their pretreatment urine scores were inadvertently not collected (2 patients) or because an iron-chelating agent that resulted in artificially colored urine was administered during the extension study (1 patient).

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Day (visit)	0*(4)	1-8	9	10	11	12	13	0*(5)	1-2	0*(9)
Urine	10	2-3	3	3	3	3	9	10	3	2
LDH	2624	–	784	–	–	697	1687	2917	–	495
AST	119	–	38	–	–	31	87	–	–	–
PK	28	–	51	–	–	35	31	23	–	51
PD	72	–	1	–	–	2	56	56	–	2

Figure 4. Relationship between complement inhibition with eculizumab and various hemolytic parameters and symptoms during a transient breakthrough in serum-complement activity. Urine color, symptoms, biochemical parameters of hemolysis, PK, and PD were assessed during a 14-day eculizumab-dosing interval in a patient with a transient breakthrough in serum hemolytic activity. Eculizumab was administered on day 0 after assay samples were collected. A urine colorimetric score of 6 or greater was considered abnormal (hemoglobinuria). Levels of the hemolytic markers LDH and AST are shown (IU/L). Eculizumab serum concentrations (PK, μg/mL) of ≥ 35 μg/mL were sufficient to maintain a serum hemolytic activity (PD, % serum hemolytic activity) of ≤ 20%, a value known to represent complete complement blockade. The eculizumab-dosing interval was reduced to 12 days between visits 5 and 9. Urine row presents colorimetric scores. — indicates not determined. *Dose of eculizumab.

Similarly, median transfusion rates decreased from 1.8 U per patient each month before treatment to 0.0 U and 0.3 U per patient each month during the first 12 weeks and the total 64 weeks of therapy, respectively ($P = .001$ for 64-week comparison in all patients). The reduction in transfusions was more pronounced in patients without cytopenia; the mean rate in this population decreased from 2.4 U per patient each month during the prestudy period to 0.2 U per patient each month during the 12- and 64-week treatment periods. Transfusion reduction in patients with poor bone marrow reserve (patients with cytopenia) decreased from a mean rate of 1.8 U per patient each month during the 1-year pretreatment period to 1.2 and 0.8 U per patient each month during the 12- and 64-week treatment periods, respectively.

Quality-of-life measurements

Quality-of-life measurements were assessed using the European Organization for Research and Treatment of Cancer (EORTC) QLQ-C30 instrument during the total 64-week treatment period and were compared with baseline values (Table 4). Significant improvements in quality-of-life domains that were demonstrated in the acute-phase study were maintained in the extension study, including global health status ($P = .009$), physical functioning ($P < .001$), emotional functioning ($P < .001$), cognitive functioning ($P = .001$), fatigue ($P < .001$), dyspnea ($P < .001$), and insomnia ($P = .031$). In addition, changes in role functioning ($P = .003$) and pain domains ($P = .023$) achieved significance during the 64-week treatment period ($P < .001$). The constipation domain showed a significant increase over the treatment period. Taken together, these results indicate that various parameters of quality of life in patients with PNH rapidly improve with eculizumab therapy and that these changes are maintained for extended lengths of time.

Table 4. Quality-of-life assessment during eculizumab treatment

Domain*	Mean baseline score†	64-wk change from baseline score‡	P§
Global health status	56.1	13.8	.009
Physical functioning	70.9	14.3	<.001
Emotional functioning	70.5	12.5	<.001
Role functioning	66.7	14.5	.003
Cognitive functioning	77.3	10.3	.001
Fatigue	47.5	−17.8	<.001
Dyspnea	39.4	−16.6	<.001
Insomnia	30.3	−8.2	.031
Pain	21.2	−8.2	.023
Constipation	3.0	4.1	<.001

*Quality of life was assessed using the European Organization for Research and Treatment of Cancer QLQ-C30 instrument.

†Mean values of linearly transformed scores.

‡Values represent least-square means. Positive change indicates improvement on Global Health Status and Functional scales, and negative change indicates improvement on Symptom scales.

§From a mixed analysis-of-covariance model with visit as a fixed effect, patient as a random effect, and baseline as a covariate.

Safety

All patients completed the extension study. No deaths occurred and no thromboses developed during the 12-month treatment period. In no patients were antibodies against eculizumab detected. All patients had at least one adverse event (AE), and one patient had a serious adverse event (SAE). No patient withdrew because of an AE. The most common AEs were flulike symptoms (4 patients), sore throat (4 patients), pain (3 patients), nausea (3 patients), bruising (3 patients), cough (3 patients), and upper respiratory infection (3 patients).

One patient experienced an SAE during the extension study characterized by neutropenia with extravascular hemolysis, which was thought to have been caused by a viral syndrome. Briefly, the patient had a 5-year history of PNH with aplastic anemia (with a normal neutrophil count) and had been on eculizumab therapy for 14 months. A viral syndrome developed, and during the next several days hemoglobin levels dropped from 10.2 to 5.3 g/dL, whereas serum LDH levels increased only slightly. The patient

Table 3. Changes in transfusion requirements during eculizumab treatment

	Transfusion rates						
	Before study*		12 wk		64 wk		
Patient group	Mean	Median	Mean	Median	Mean	Median	P†
All patients	2.1	1.8	0.6	0.0	0.5	0.3	.001
Patients without cytopenia‡	2.4	2.0	0.2	0.0	0.2	0.1	–
Patients with cytopenia§	1.8	1.8	1.2	0.7	0.8	0.7	–

Transfusion rates are units per patient per month.

– indicates not determined.

*Values during 52-week period before treatment.

†Comparisons of median change from before study to the 64-week treatment period.

‡Platelet count ≥ 150×10^9/L; n = 6.

§Platelet count < 150×10^9/L; n = 5.

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received a transfusion of 4 U PBRCs; at the next study visit, the hemoglobin level had returned to 10.7 g/dL and the platelet count was normal, but the neutrophil count was abnormally low (260/μL). Two weeks later, the neutrophil count had recovered to almost-normal levels (1600/μL). The investigator did not think the SAE was caused by the study medication. The patient remained transfusion independent for the remainder of the extension study.

Discussion

We reported previously that eculizumab therapy in patients with PNH resulted in a dramatic reduction in hemolysis and transfusion requirements. The extension study was conducted to obtain long-term data regarding eculizumab use in this patient population. Here we report the sustained efficacy and safety of eculizumab in a 1-year, open-label extension study.

Serum hemolytic activity in 9 of 11 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. One patient had a breakthrough of serum hemolytic activity during the acute-phase study, and another patient broke through early in the extension study. PK analysis of both breakthrough patients demonstrated a simultaneous decrease in eculizumab level below that required to completely block complement activity (≥ 35 μg/mL).[9] Adjustment of the eculizumab dosing interval in these 2 patients from every 14 days to every 12 days successfully sustained trough levels of eculizumab higher than 35 μg/mL and consistently blocked serum hemolytic activity for the remainder of the extension study. The effective and consistent blockade of complement achieved with the 12-day dosing interval was supported by the resolution of symptoms, including hemoglobinuria and dysphagia, and lower levels of LDH and AST (Figure 4). Taken together, these data illustrate the tight relationship between complement blockade, hemolysis, and symptoms in PNH.

The dramatic reduction in LDH levels that was demonstrated immediately on the administration of eculizumab in the initial study was sustained during the extension study, providing strong evidence that eculizumab effectively and durably inhibits intravascular hemolysis in patients with PNH. LDH levels were reduced from a mean of 3110.7 IU/L during the 52-week period before treatment to a mean of 622.4 IU/L during the 64 weeks of eculizumab therapy. In addition, detailed analysis of PK and PD demonstrated a strong correlation between the return of complement activity, hemolysis, and an increase in LDH levels (Figure 4). These data confirm that LDH levels can be used as an accurate measure of intravascular hemolysis in PNH, and they provide evidence that effective complement blockade during eculizumab therapy can be determined by monitoring levels of this enzyme.

LDH levels remained slightly elevated in most patients during eculizumab treatment, suggesting low levels of ongoing hemolysis. Undetectable haptoglobin levels and elevated bilirubin levels also support residual hemolysis in the midst of terminal complement inhibition. This may be attributed to yet undefined, noncomplement-mediated mechanisms of PNH RBC clearance, as recently described.[11] Alternatively, it is possible that a fraction of PNH RBCs is cleared through complement-mediated events before C5, such as a C3b coating and clearance. In this regard, slightly elevated levels of LDH have also been reported in a patient with coexistent PNH and C9 deficiency.[12]

The marked reduction in transfusions demonstrated in the initial eculizumab study was sustained in the extension study. Further, patients who had good bone marrow reserve (those without cytopenia) showed greater reductions in transfusion requirements than did patients who had hypoplasia (those with cytopenia). Importantly, 3 patients with hypoplasia who received transfusions during the acute-phase study were subsequently treated with erythropoietin during the extension study. Two of the 3 patients responded with an increase in reticulocyte counts and a further reduction in transfusion requirements (data not shown). These data suggest that erythropoietin therapy in PNH patients with hypoplasia increases erythropoiesis and that concomitant eculizumab therapy protects against increased hemolysis, with a resultant improvement in response. Three of 11 PNH patients remain transfusion independent after 64 weeks of eculizumab therapy.

Interestingly, eculizumab therapy resulted in dramatic reductions in transfusion requirements even though hemoglobin levels and reticulocyte counts frequently remained fairly constant. However, before eculizumab treatment, hemoglobin levels in patients were artificially maintained through regular transfusions of packed RBCs. Thus, stabilization of hemoglobin levels with a concomitant reduction in or cessation of transfusions likely represents a net increase in hemoglobin levels. These data suggest that the resolution of hemolysis in patients with PNH results in a new steady state hemoglobin level, determined by a combination of the extent of the underlying bone marrow dysfunction, the size of the type III RBC clone, and the new level (if any) of transfusion requirement.

There was a sustained improvement in quality of life for transfusion-dependent PNH patients administered eculizumab, as measured by the EORTC QLQ-C30. Quality-of-life parameters that showed significant improvement include global health status, physical functioning, emotional functioning, role functioning, cognitive functioning, fatigue, dyspnea, insomnia, and pain. Improvements in quality-of-life parameters in PNH patients taking eculizumab will be further investigated in subsequent placebo-controlled trials.

Clinical assessment of PNH symptoms not captured by the QLQ-C30 instrument, such as dysphagia, abdominal pain, and erectile failure, showed complete resolution, or at least dramatic improvement, during eculizumab treatment.[13] These symptoms have been shown to correlate with a large PNH type III clone size, suggesting that they are related to excessive hemolysis.[14] In addition, these symptoms have been attributed to smooth muscle dystonia caused by the scavenging of nitric oxide by free plasma hemoglobin.[14-18] The capacity of free plasma hemoglobin to scavenge nitric oxide during intravascular hemolysis has been demonstrated in patients with sickle cell anemia.[19] The relationship between levels of free plasma hemoglobin and nitric oxide in patients with PNH remains to be elucidated.

Thrombosis accounts for most deaths in patients with PNH. Studies have reported a strong correlation between a large PNH type III neutrophil clone and the occurrence of thrombosis.[14,20] Hall et al[20] reported that in approximately 44% of patients with large PNH clones, venous thrombosis developed in the first 10 years after diagnosis. The tendency toward thrombosis in patients with PNH is thought to be multifactorial in etiology, involving both the absence of GPI-anchored complement inhibitors on the surfaces of circulating platelets and the high levels of intravascular free plasma hemoglobin with the consequent scavenging of nitric oxide.[18,21-25] It is reasonable to hypothesize that thrombosis resulting from either or both of these mechanisms should be reduced by terminal complement blockade. Although no thrombotic events occurred

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during eculizumab treatment, 6 of the 11 patients were on warfarin therapy before and during the study. Further analyses and studies are required to investigate whether eculizumab reduces the risk for thrombosis in patients with PNH.

Eculizumab continued to be safe and well tolerated in the PNH extension study. No deaths occurred and no thromboses developed in this study. Each patient had at least one AE; flulike symptoms and sore throat were the most common. Evaluation of AEs in other placebo-controlled eculizumab studies suggests that the reported AEs in the PNH extension study were similar in type and frequency to those reported in other placebo treatment groups. The single SAE, transient neutropenia, was not thought by the principal investigator to be related to study medication; rather, it was thought to be the result of a viral syndrome. All patients chose to continue treatment in a second extension study, and evaluation of eculizumab therapy in a broader population of PNH patients is under way.

Results of this 1-year extension study showed that eculizumab therapy continues to be safe and well tolerated in PNH patients. Additionally, long-term complement inhibition with eculizumab in this patient population has resulted in sustained reductions in hemolysis and blood transfusions and continued improvement in quality of life.

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Sustained response and long-term safety of eculizumab in paroxysmal nocturnal hemoglobinuria

Anita Hill, Peter Hillmen, Stephen J. Richards, Dupe Elebute, Judith C. Marsh, Jason Chan, Christopher F. Mojcik and Russell P. Rother

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Submission number	5510715
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Patent number	EP2359834
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Your reference	AMG17203OP
Opponent	Amgen Inc.
Title	Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement

Documents submitted

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European patent 2 359 834 (Application No. 11 001 632.6)
Title: Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement - EP 2 359 834
Proprietor: Alexion Pharmaceuticals, Inc.
Opponent: Amgen Inc.
Our Ref.: AMG17203OP

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(54) **Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement**

Hemmer des Komplements zur Behandlung der paroxysmalen nächtlichen Haemoglobinurie

Traitement des patients souffrant de l'hémoglobinurie paroxystique nocturne par un inhibiteur complémentaire

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- **HILLMEN P ET AL: "Eculizumab, a C5 complement blocking antibody, is the first therapy to reduce transfusion requirements in patients with paroxysmal nocturnal haemoglobinuria (PNH)", BRITISH JOURNAL OF HAEMATOLOGY, WILEY-BLACKWELL PUBLISHING LTD, GB, vol. 121, no. Suppl.1, 1 May 2003 (2003-05-01), page 87, XP009144758, ISSN: 0007-1048**
- **HILLMEN P ET AL: "EFFECT OF ECULIZUMAB ON HEMOLYSIS AND TRANSFUSION REQUIREMENTS IN PATIENTS WITH PAROXYSMAL NOCTRURNAL HEMAGLOBINURIA", NEW ENGLAND JOURNAL OF MEDICINE, MASSACHUSETTS MEDICAL SOCIETY, BOSTON, MA, US, vol. 350, no. 6, 5 February 2004 (2004-02-05), pages 552-559, XP009057948, ISSN: 1533-4406, DOI: DOI:10.1056/NEJMOA031688**
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- DATABASE MEDLINE [Online] US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US October 1992 MORRIS J T ET AL: 'Recurrence of neisserial meningococcemia due to deficiency of terminal complement component.' Database accession no. NLM1411724 & MORRIS J T ET AL: "Recurrence of neisserial meningococcemia due to deficiency of terminal complement component.", SOUTHERN MEDICAL JOURNAL OCT 1992, vol. 85, no. 10, October 1992 (1992-10), pages 1030-1031, ISSN: 0038-4348
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Description

BACKGROUND

[0001] Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hematologic disease that results from clonal expansion of hematopoietic stem cells with somatic mutations in the X-linked gene called PIG-A.[1,2] Mutations in PIG-A lead to an early block in the synthesis of glycosylphosphatidylinositol (GPI)-anchors, which are required to tether many proteins to the cell surface. Consequently, PNH blood cells have a partial (type II) or complete (type III) deficiency of GPI-anchored proteins.

[0002] Intravascular hemolysis is a prominent feature of PNH and a direct result of the absence of the GPI-anchored complement regulatory protein CD59.[3,4] Under normal circumstances, CD59 blocks the formation of the terminal complement complex (also called the membrane attack complex) on the cell surface, thereby preventing erythrocyte lysis and platelet activation.[5-8] Excessive or persistent intravascular hemolysis in PNH patients not only results in anemia (normal ranges of hemoglobin are 14-18 g/dL for men and 12-16 g/dL for women, and persons with lower levels are considered to be anemic), but also hemoglobinuria and clinical sequelae related to the release of the erythrocyte contents into the circulation: fatigue, thrombosis, abdominal pain, dysphagia, erectile dysfunction, and pulmonary hypertension.[9,10,21,22] Indeed, impaired quality of life in PNH is disproportionate to the degree of anemia. Many PNH patients depend on blood transfusions to maintain adequate erythrocyte hemoglobin levels. There have been no therapies that effectively reduce intravascular hemolysis and improve the associated clinical morbidities in PNH.

[0003] Eculizumab is a humanized monoclonal antibody directed against the terminal complement protein C5.[11] In a preliminary, 12-week, open-label clinical study in 11 PNH patients, eculizumab was shown to reduce intravascular hemolysis and transfusion requirements.[12] However, this unblinded study involved a small number of patients with no control arm and without protocol-driven transfusion standards.

SUMMARY

[0004] The present invention is characterised by the claim.

[0005] The present pivotal, phase III study, Transfusion Reduction Efficacy and Safety Clinical Investigation, Randomized, Multi-Center, Double-Blind, Placebo-Controlled, Using Eculizumab in Paroxysmal Nocturnal Hemoglobinuria (TRIUMPH), evaluated the effect of eculizumab on the stabilization of hemoglobin levels and transfusion requirements during 6 months of treatment in a cohort of 87 transfusion-dependent PNH patients. Measures of intravascular hemolysis and quality of life were also assessed. This is the first placebo controlled study of a PNH patient population to control hemolysis and to differentiate between the effects due to hemolysis and the effects due to anemia.

[0006] It has been surprisingly discovered that certain aspects of quality of life were unexpectedly improved by the treatment of PNH patients with eculizumab. Furthermore, these improvements in the quality of life were independent of transfusion. The improved aspects include, e.g., global health status, physical functioning, emotional functioning, cognitive functioning, role functioning, social functioning, fatigue, pain, dyspnea, appetite loss and insomnia. Improvement was also seen in nausea and vomiting, diarrhea, constipation, and financial difficulties but did not reach the level of statistical significance. Because the treated patients remained anemic throughout their treatment, it was unexpected that all of these improvements would have been seen because they were previously thought to be a result of the patient being anemic. Although not wishing to be bound by any theory, it appears that some of the symptoms are likely due, at least in part, to hemolysis and release of hemoglobin into the bloodstream and do not result solely from the patient being anemic. The treatment with eculizumab decreases the amount of lysis thereby limiting hemoglobin release into the bloodstream, thereby apparently resulting in the improvements seen in the treated patients' quality of life. The results presented herein indicate that any treatment that decreases hemolysis in a patient will result in an improvement in the quality of life of said patient.

[0007] Described is a method to improve at least one aspect
of the quality of life of a patient suffering from paroxysmal nocturnal hemoglobinuria, said method comprising administering to said patient in need thereof a compound which inhibits complement or inhibits formation of C5b-9.

[0008] Described is a method to improve at least one aspect
of the quality of life of a patient suffering from paroxysmal nocturnal hemoglobinuria, said method comprising administering to said patient in need thereof a compound which inhibits intravascular hemolysis. Said method may result 2 in a greater than 30% reduction in LDH in said patient.

[0009] Described is a method to improve at least one aspect
of the quality of life of an anemic patient whose anemia results at least in part from hemolysis, said method comprising administering to said patient in need thereof a compound which inhibits intravascular hemolysis, wherein said patient remains anemic. Said method may result in a greater than 30% reduction in LDH in said patient.

[0010] Described is a method of prolonging the health-adjusted life expectancy of a patient comprising administering

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to said patient in need thereof a compound which inhibits formation of C5b-9. Said patient may be anemic. Said patient may remain anemic following
treatment. Said patient may have a hemoglobin level less than i) 14 g/dL
if a man or ii) 12 g/dL if a woman. Said patient may have a hemoglobin
level less than i) 13 g/dL if a man or ii) 11 g/dL if a woman. Said patient may have a hemoglobin level less than i) 12 g/dL if a man or ii) 10 g/dL if a woman. Said patient suffers from paroxysmal nocturnal hemoglobinuria.

[0011] The application provides a pharmaceutical composition comprising an antibody that binds C5 or an active antibody fragment thereof as characterised in the claim. The antibody that binds C5 or an active antibody fragment thereof is eculizumab. The pharmaceutical formulations of the application may be administered to a subject having PNH.

[0012] Described are kits comprising a pharmaceutical composition of the application. The kit may further comprise at least
one component of a closed sterile system. Components of the closed sterile system include, but are not limited to, needles, syringes, catheter based syringes, needle based injection devices, needle-less injection devices, filters, tubing, valves and cannulas. The kit may comprise components for the removal of a preservative from the composition. Such components include filters, syringes, vials, containers, tubing, etc.

[0013] Said quality of life may be measured by a FACIT-Fatigue score.

[0014] The FACIT-Fatigue score may increase by at least 3 points. The FACIT-Fatigue score may increase by ≥ 4 points.

[0015] Said quality of life may be measured by an EORTC QLQ-C30 score. Said EORTC QLQ-C30 score may improve by ≥ 10% of the pretreatment score. Said aspect of the quality of life as measured by an EORTC QLQ-C30 score may be selected from the group consisting of a) global health status, b) physical functioning, c) emotional functioning, d) cognitive functioning, e) role functioning, f) social functioning, g) fatigue, h) pain, i) dyspnea, j) appetite loss, and k) insomnia. Said aspect of quality of life may be fatigue.

[0016] Said antibody binds C5 and is eculizumab. Said antibody may be administered for at least 6 months. Said patient may have aplastic anemia or myelodysplastic syndrome.

[0017] Said antibody that binds C5 is administered in a single unit dosage form. The single unit dosage form is a 300 mg unit dosage form. The single unit dosage form is a sterile solution. The single unit dosage form is a preservative free formulation. The 300 mg single-use dosage form comprises 30 ml of a 10 mg/ml sterile, preservative free solution.

[0018] In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof comprises a heavy chain variable region and a light chain variable region, wherein the heavy chain variable region comprises one or more CDR regions having an amino acid sequence selected from the group consisting of SEQ ID NO:5, SEQ ID NO:6, or SEQ ID NO:7, and wherein the light chain variable region comprises one or more CDR regions having an amino acid sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10. In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof comprises a heavy chain variable region and a light chain variable region, wherein the heavy chain variable region consists of SEQ ID NO: 1 and the light chain variable region consists of SEQ ID NO: 3. The pharmaceutical composition comprises eculizumab. In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof comprises a heavy chain and a light chain, wherein the heavy chain consists of SEQ ID NO: 2 and the light chain consists of SEQ TD NO: 4.

[0019] Said patient may be anemic. Said patient may remain anemic following treatment. Said patient may have a hemoglobin level less than i) 14 g/dL if a man or ii) 12 g/dL if a woman. Said patient may have a hemoglobin level less than i) 13 g/dL if a man or ii)) 11 g/dL if a woman. Said patient may have a hemoglobin level less than i) 12 g/dL if a man or ii) 10 g/dL if a woman.

[0020] Said health-adjusted life expectancy may be measured according to a unit selected from the group consisting of Years of potential life lost, Disability-free life expectancy, Health-adjusted life year, Quality adjusted life year, Healthy years equivalents, Healthy days gained, Episode-free day, Q-TWiST, Health Utilities Index, or Years of healthy life.

[0021] The health-adjusted life expectancy in a subject may be prolonged by at least one day. The health-adjusted life expectancy in a subject may be prolonged by at least one week. The health-adjusted life expectancy in a subject may be prolonged by at least one month. The health-adjusted life expectancy in a subject may be prolonged by at least one year.

[0022] The pharmaceutical composition is in a single unit dosage form. The single unit dosage form is a 300 mg unit dosage form. The pharmaceutical composition is a sterile solution.

[0023] Figure 2 shows the effect of eculizumab treatment on transfusion requirements in PNH patients. This is a Kaplan-Meier plot of time to first transfusion for eculizumab- and placebo-treated patients from baseline through week 26. The P value is from the log rank analysis.

[0024] Figure 3 shows the effect of eculizumab on fatigue assessed by the FACIT-Fatigue Instrument. Quality of Life scores were assessed using the Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT-Fatigue) instrument. Values for change from baseline to 26 weeks represent least-square means. A positive change indicates an improvement and a negative change indicates deterioration in the FACIT-Fatigue measures of quality of life.

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DETAILED DESCRIPTION

I. Definitions

[0025] The term "derived from" means "obtained from" or "produced by" or "descending from".

[0026] The term "genetically altered antibodies" means antibodies wherein the amino acid sequence has been varied from that of a native antibody. Because of the relevance of recombinant DNA techniques to this application, one need not be confined to the sequences of amino acids found in natural antibodies; antibodies can be redesigned to obtain desired characteristics. The possible variations are many and range from the changing of just one or a few amino acids to the complete redesign of, for example, the variable or constant region. Changes in the constant region will, in general, be made in order to improve or alter characteristics, such as complement fixation, interaction with membranes and other effector functions. Changes in the variable region will be made in order to improve the antigen binding characteristics.

[0027] The term "an antigen-binding fragment of an antibody" refers to any portion of an antibody that retains the binding utility to the antigen. An exemplary antigen-binding fragment of an antibody is the heavy chain and/or light chain CDR, or the heavy and/or light chain variable region.

[0028] The term "homologous," in the context of two nucleic acids or polypeptides refers to two or more sequences or subsequences that have at least about 85%, at least 90%, at least 95%, or higher nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using the following sequence comparison method and/or by visual inspection. In certain embodiments, the "homolog" exists over a region of the sequences that is about 50 residues in length, at least about 100 residues, at least about 150 residues, or over the full length of the two sequences to be compared.

[0029] Methods of determining percent identity are known in the art. "Percent (%) sequence identity" with respect to a specified subject sequence, or a specified portion thereof, may be defined as the percentage of nucleotides or amino acids in the candidate derivative sequence identical with the nucleotides or amino acids in the subject sequence (or specified portion thereof), after aligning the sequences and introducing gaps, if necessary to achieve the maximum percent sequence identity, as generated by the program WU-BLAST-2.0a19 (Altschul et al., J. Mol. Biol. 215:403-410 (1997); http://blast.wustl.edu/blast/README.htm-1) with search parameters set to default values. The HSP S and HSP S2 parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched. A "% identity value" is determined by the number of matching identical nucleotides or amino acids divided by the sequence length for which the percent identity is being reported.

II. Overview

[0030] The present disclosure relates to a pharmaceutical composition as characterised in the claim. It is for treating paroxysmal nocturnal hemoglobinuria ("PNH"), more specifically to improving certain aspects of quality of life which are impaired in PNH patients. Specifically, the pharmaceutical composition for treating PNH which is described herein, involve using eculizumab which binds to or otherwise blocks the generation and/or activity of C5. The present invention has been found to provide surprising results. For instance, hemolysis rapidly ceases upon administration of the compound which binds to or otherwise blocks the generation and/or activity of said complement component, with hemoglobinuria being significantly reduced after treatment. Also, hemolytic patients can be rendered less dependent on transfusions or transfusion-independent for extended periods (twelve months or more), well beyond the 120 day life cycle of red blood cells. In addition, type III red blood cell count can be increased dramatically in the midst of other mechanisms of red blood cell lysis (non-complement mediated and/or earlier complement component mediated e.g., Cb3). Another example of a surprising result is that symptoms resolved, indicating that NO serum levels were increased enough even in the presence of other mechanisms of red blood cell lysis. These and other results reported herein are unexpected and could not be predicted from prior treatments of hemolytic diseases.

III The Complement System

[0031] The complement system, useful complement inhibitors, and use of these inhibitors to treat PNH and other patients are more fully described in PCT Patent Application PCT/US2005/003225 filed February 3, 2005 and published as International Publication Number WO 2005/074607 A2 on August 18, 2005.

[0032] 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

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the production of products with opsonic, immunoregulatory, and lytic functions.

[0033] 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.

[0034] 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.

[0035] C3a is an anaphylatoxin. C3b binds to bacterial and other cells, as well as to certain viruses and immune complexes, and tags them for removal from the circulation. C3b in this role is known as opsonin. The opsonic function of C3b is considered to be the most important anti-infective action of the complement system. Patients with genetic lesions that block C3b function are prone to infection by a broad variety of pathogenic organisms, while patients with lesions later in the complement cascade sequence, i.e., patients with lesions that block C5 functions, are found to be more prone only to Neisseria infection, and then only somewhat more prone (Fearon, 1983).

[0036] C3b also forms a complex with other components unique to each pathway to form classical or alternative C5 convertase, which cleaves C5 into C5a and C5b. C3 is thus regarded as the central protein in the complement reaction sequence since it is essential to both the alternative and classical pathways (Wurzner et al., 1991). This property of C3b is regulated by the serum protease Factor I, which acts on C3b to produce iC3b. While still functional as opsonin, iC3b cannot form an active C5 convertase.

[0037] C5 is a 190 kDa beta globulin found in normal serum at approximately 75 ng/mL (0.4 μM). C5 is glycosylated, with about 1.5-3 percent of its mass attributed to carbohydrate. Mature C5 is a heterodimer of a 999 amino acid 115 kDa alpha chain that is disulfide linked to a 656 amino acid 75 kDa beta chain. C5 is synthesized as a single chain precursor protein product of a single copy gene (Haviland et al., 1991). The cDNA sequence of the transcript of this gene predicts a secreted pro-C5 precursor of 1659 amino acids along with an 18 amino acid leader sequence.

[0038] The pro-C5 precursor is cleaved after amino acid 655 and 659, to yield the beta chain as an amino terminal fragment (amino acid residues +1 to 655) and the alpha chain as a carboxyl terminal fragment (amino acid residues 660 to 1658), with four amino acids deleted between the two.

[0039] C5a is cleaved from the alpha chain of C5 by either alternative or classical C5 convertase as an amino terminal fragment comprising the first 74 amino acids of the alpha chain (i.e., amino acid residues 660-733). Approximately 20 percent of the 11 kDa mass of C5a is attributed to carbohydrate. The cleavage site for convertase action is at or immediately adjacent to amino acid residue 733. A compound that would bind at or adjacent to this cleavage site would have the potential to block access of the C5 convertase enzymes to the cleavage site and thereby act as a complement inhibitor.

[0040] C5 can also be activated by means other than C5 convertase activity. Limited trypsin digestion (Minta and Man, 1977; Wetsel and Kolb, 1982) and acid treatment (Yamamoto and Gewurz, 1978; Vogt et al., 1989) can also cleave C5 and produce active C5b.

[0041] C5a is another anaphylatoxin. C5b combines with C6, C7, and C8 to form the C5b-8 complex at the surface of the target cell. Upon binding of several C9 molecules, the membrane attack complex (MAC, C5b-9, terminal complement complex-TCC) is formed. When sufficient numbers of MACs insert into target cell membranes the openings they create (MAC pores) mediate rapid osmotic lysis of the target cells. Lower, non-lytic concentrations of MACs can produce other effects. In particular, membrane insertion of small numbers of the C5b-9 complexes into endothelial cells and platelets can cause deleterious cell activation. In some cases activation may precede cell lysis.

[0042] 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.

[0043] The beneficial effect of anti-C5 mAb has previously been reported in several experimental models including myocardial reperfusion (Vakeva et al., 1998), systemic lupus erythematosus (Wang et al., 1996) and rheumatoid arthritis (Wang et al., 1995); as well as in human clinical trials (Kirschfink, 2001) of autoimmune disease, cardiopulmonary bypass and acute myocardial infarction.

IV Measures of Quality ofLife

[0044] Various measurements exist to assess quality of life and the effect of medical interventions on quality of life for example the Mini-Mental State Examination (MMSE), the Short Test of Mental Status, the European Organization for Research and Treatment of Cancer (EORTC) Quality of Life Questionnaire, the FACIT questionnaires and subscales

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including fatigue and anemia, the Likert Scale, and Borg Scale (Tombaugh, et al., J. Am. Geriatr. Soc. 40:922, 1992; Cummings, JAMA. 269(18):2420, 1993; Crum, et al., JAMA. 269(18):2386, 1993; Folstein, et al., J. Psychiat. Res. 12:189,1975; Kokmen, et al., Mayo Clin. Proc. 62:281, 1987; Tang-Wai, et al., Arch. Neurol. 60:1777, 2003; Tamburini, Ann. Oncol. 12(Suppl. 3):S7, 2001; Webster et al., Health and Quality of Life Outcomes. 1:79, 2003, www.hqlo.com/content/l/l/79; Grant, et al., Chest. 116:1208, 1999; and www.qolid.org). Any of these measurements may be used to assess the change in quality of life due to administration of a compound which inhibits complement or inhibits formation of C5b-9.

[0045] Improvement in quality of life due to administration of a compound which inhibits complement or inhibits formation of C5b-9 may be measured by the Functional Assessment of Chronic Illness Therapy (FACIT) Measurement System. Improvement in quality of life may be measured by: a) full scales; b) stand-alone subscales; and c) symptom indices.

[0046] Improvement in quality of life due to administration of a compound which inhibits complement or inhibits formation of C5b-9 may be measured by a

European Organization for Research and Treatment of Cancer (EORTC) Quality of Life Questionnaire. In certain embodiments, the EORTC questionnaire is the QLQ-C30.

[0047] Improvement in quality of life may be measured by the health-adjusted life expectancy (HALE) index as described in Wilkins, R. and Adams, OB., Am J Public Health, 73:1073-1080 (1983). Health-adjusted life expectancy is an average of the quality-adjusted life years (QALY) for a given population and can be used to evaluate the therapeutic value of a medical intervention. Quality-adjusted life years is a health index that weighs each year of life on a scale from 1 to 0 (Weinstein MC and Stason WB, N Engl J Med, 296:716-721 (1977)). Perfect health is rated as 1, death is rated as 0, and disability and pain are rated based on severity. QALY is determined by multiplying the number of years at each health status.

[0048] Improvement in quality of life may be measured by the following instruments: Years of potential life lost, Disability-free life expectancy, Health-adjusted life year, Quality adjusted life year, Healthy years equivalents, Healthy days gained, Episode-free day, Q-TWiST, Health Utilities Index, and Years of healthy life. These measurements account for both changes in mortality as well as changes in morbidity and disability. Any of these measurements may be used to assess the change in quality of life due to administration of a compound which inhibits complement or inhibits formation of C5b-9.

[0049] The disclosed methods may improve the quality of life of a patient for at least one day, at least one week, at least two weeks, at least three weeks, at least one month, at least two months, at least three months, at least 6 months, at least one year, at least 18 months, at least two years, at least 30 months, or at least three years, or the duration of treatment.

[0050] The symptoms used to measure quality of life may be scaled for intensity. The symptoms may be scaled for frequency. The symptoms may be scaled for intensity and frequency.

[0051] Described is a method for prolonging the health-adjusted life expectancy of a subject comprising administering to the subject a compound which inhibits complement or inhibits formation of C5b-9. The above measurements account for both changes in mortality as well as changes in morbidity and disability. Any of these measurements may be used to assess the change in quality-adjusted life expectancy due to administration of a compound which inhibits complement or inhibits formation of C5b-9.

[0052] The disclosed methods may prolong the health-adjusted life

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

V Inhibitors of the Complement Cascade

[0053] The concentration and/or physiologic activity of C5a and C5b in a body fluid can be measured by methods well known in the art. For C5a such methods include chemotaxis assays, RIAs, or ELISAs (see, for example, Ward and Zvaifler, J Clin Invest. 1971 Mar;50(3):606-16; Wurzner, et al., Complement Inflamm. 8:328-340, 1991). For C5b, hemolytic assays or assays for soluble C5b-9 as discussed herein can be used. Other assays known in the art can also be used.

[0054] The described method of inhibiting complement activity is to use a monoclonal antibody which binds to complement C5 and inhibits cleavage. This decreases the formation of both C5a and C5b while at the same time allowing the formation of C3a and C3b which are beneficial to the recipient. The antibody is disclosed in U.S. Patent 6,355,245 and is named eculizumab. A similar antibody against mouse C5 is called BB5.1 (Frei et al., Mol. Cell. Probes. 1:141-149 (1987)).

[0055] It is well known that the binding to a molecule (or a pathogen) of antibodies with an Fc region assists in the

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

VI Methods of Treatment

[0056] Methods described in the application are used to treat paroxysmal nocturnal hemoglobinuria associated symptoms. Treatments of the application may be used in combination with known treatments for paroxysmal nocturnal hemoglobinuria. Treatments of the application may be co-administered with other treatments that treat symptoms of paroxysmal nocturnal hemoglobinuri a.

VII Pharmaceutical Formulations and Uses

[0057] The pharmaceutical composition is in a single unit dosage form, namely a 300 mg unit dosage form. The pharmaceutical composition is a sterile solution. The pharmaceutical composition is a preservative free formulation. The pharmaceutical composition comprises a 300 mg single-use formulation of 30 ml of a 10 mg/ml sterile, preservative free solution. The antibody may be administered according to the following protocol:

> 600 mg via 25 to 45 minute IV infusion every 7 ± 2 days for the first 4 weeks, followed by 900 mg for the fifth dose 7 ± 2 days later, then 900 mg every 14 ± 2 days thereafter. Antibody is administered via IV infusion over 25 to 45 minutes.

[0058] Administration of the anti-C5 antibodies will generally be performed by an intravascular route, e.g., via intravenous infusion by injection. Other routes of administration may be used if desired but an intravenous route will be the most preferable. The formulations may contain pharmaceutically acceptable auxiliary substances as required, such as, tonicity adjusting agents, wetting agents, bactericidal agents, preservatives, stabilizers, and the like. Eculizumab may be administered via

[0059] A typical therapeutic treatment includes a series of doses, which will usually be administered concurrently with the monitoring of clinical endpoints with the dosage levels adjusted as needed to achieve the desired clinical outcome. Treatment may be administered in multiple dosages over at least a week. Treatment may be administered in multiple dosages over at least a month. Treatment may be administered in multiple dosages over at least a year. Treatment may be administered in multiple dosages over the remainder of the patient's life.

[0060] The frequency of administration may also be adjusted according to various parameters. These include the clinical response, and the levels of the antibody in a body fluid, such as, blood, plasma, serum, or synovial fluid. To guide adjustment of the frequency of administration, levels of the therapeutic of the disclosure in the body fluid may be monitored during the course of treatment.

[0061] The frequency of administration may be adjusted according to an assay measuring cell-lysing ability of complement present in one or more of the patient's body fluids. The cell-lysing ability can be measured as percent hemolysis in hemolytic assays of the types described herein. A 10% or 25% or 50% reduction in the cell-lysing ability of complement present in a body fluid after treatment with the antibody capable of inhibiting complement used in the practice of the application means that the percent hemolysis after treatment is 90, 75, or 50 percent, respectively, of the percent hemolysis before treatment.

[0062] For the treatment of PNH by systemic administration of eculizumab (as opposed to local administration), administration of a large initial dose is specific, i.e., a single initial dose sufficient to yield a substantial reduction, and more preferably an at least about 50% reduction, in the hemolytic activity of the patient's serum. Such a large initial dose is preferably followed by regularly repeated administration of tapered doses as needed to maintain substantial reductions of serum hemolytic titer. The initial dose may be given by both local and systemic routes, followed by repeated systemic administration of tapered doses as described above.

[0063] Formulations particularly useful for antibody-based therapeutic agents are also described in U.S. Patent App. Publication Nos. 20030202972, 20040091490 and 20050158316. The liquid formulations of the application may be substantially free of surfactant and/or inorganic salts. The liquid formulations may have a pH ranging from about 5.0 to about 7.0. The liquid formulations may comprise histidine at a concentration ranging from about 1 mM to about 100 mM. The liquid formulations may comprise histidine at a concentration ranging from 1 mM to 100 mM. It is also contemplated that the liquid formulations may further comprise one or more excipients such as a saccharide, an amino acid (e.g., arginine, lysine, and methionine) and a polyol. Additional descriptions and methods of preparing and analyzing liquid formulations can be found, for example, in PCT publications WO 03/106644, WO 04/066957, and WO 04/091658.

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[0064] Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the pharmaceutical compositions of the application.

[0065] In certain embodiments, formulations of the subject antibodies are pyrogen-free formulations which are substantially free of endotoxins and/or related pyrogenic substances. Endotoxins include toxins that are confined inside microorganisms and are released when the microorganisms are broken down or die. Pyrogenic substances also include fever-inducing, thermostable substances (glycoproteins) from the outer membrane of bacteria and other microorganisms. Both of these substances can cause fever, hypotension and shock if administered to humans. Due to the potential harmful effects, it is advantageous to remove even low amounts of endotoxins from intravenously administered pharmaceutical drug solutions. The Food & Drug Administration ("FDA") has set an upper limit of 5 endotoxin units (EU) per dose per kilogram body weight in a single one hour period for intravenous drug applications (The United States Pharmacopeial Convention, Pharmacopeial Forum 26 (1):223 (2000)). When therapeutic proteins are administered in amounts of several hundred or thousand milligrams per kilogram body weight, as can be the case with monoclonal antibodies, it is advantageous to remove even trace amounts of endotoxin.

[0066] Doses of therapeutics of the disclosure will also vary depending on the manner of administration, the particular symptoms of the patient being treated, the overall health, condition, size, and age of the patient, and the judgment of the prescribing physician.

[0067] The formulations of the application can be distributed as articles of manufacture comprising packaging material and a pharmaceutical agent which comprises the antibody capable of inhibiting complement and a pharmaceutically acceptable carrier as appropriate to the mode of administration. The packaging material may include a label which indicates that the formulation is for use in the treatment of PNH.

[0068] The pharmaceutical formulations and uses of the disclosure may be combined with any known complement inhibitors or hemolytic diseases treatments known in the art.

[0069] The application describes kits comprising a pharmaceutical composition of the application. The kit may further comprise at least one component of a closed sterile system. Components of the closed sterile system include, but are not limited to, needles, syringes, catheter based syringes, needle based injection devices, needle-less injection devices, filters, tubing, valves and cannulas. The kit may comprise components for the removal of a preservative from the composition. Such components include filters, syringes, vials, containers, tubing, etc.

EXEMPLIFICATION

METHODS

Patient Selection

[0070] The TRIUMPH trial consisted of a 2-week screening period, an observation period of up to 3 months duration, and a 26-week treatment period.

[0071] During the screening period, patients were evaluated with respect to inclusion and exclusion criteria. Men and women, 18 years or older, diagnosed as having PNH with a type III erythrocyte population of ≥10%, and who had received at least 4 transfusions in the previous 12 months were eligible. Concomitant administration of erythropoietin, immunosuppressants, corticosteroids, coumadin, low molecular weight heparin, iron supplements, and folic acid were not reasons for exclusion, provided the doses were steady prior to the first visit and throughout the duration of the study. Because of the increased frequency of neisserial infections in individuals genetically deficient in terminal complement proteins, all patients were vaccinated against *Neisseria meningitides*. Patients were to avoid conception. The protocol was approved by an Investigational Review Board at each clinical site and written informed consent was obtained from all patients enrolled.

[0072] Patients transfused with a mean pre-transfusion hemoglobin level >10.5 g/dL over the previous 12 months, and those who showed evidence of having a suppressed immune response, complement deficiency, or active bacterial infection, including any history of meningococcal disease, were excluded from the study. Patients were also not eligible if they had previously received a bone marrow transplant or if they had participated in another trial or received another investigational drug within 30 days of the first visit. An individualized transfusion algorithm was calculated for each patient based on their prior 12-month transfusion history; the written algorithm documented the number of packed red blood cell (PRBC) units transfused for given hemoglobin values and served as a prospectively determined guide for transfusion during observation and treatment periods.

[0073] Each patient considered eligible entered an observation period of up to 13 weeks in order to confirm their PBRC transfusion dependence. At least one transfusion --termed the "qualifying" transfusion-- during the 13-week observation period at a hemoglobin value at or below 9 g/dL with symptoms, or at or below 7 g/dL with or without symptoms, in

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

Study Design

[0074] Patients were randomly assigned on a one-on-one basis to receive either placebo or eculizumab (Soliris™, Alexion Pharmaceuticals, Inc.) within 10 days of the qualifying transfusion. Study medication was dosed in a blinded fashion as follows: 600 mg eculizumab for patients randomly assigned to active drug, or placebo for those patients randomly assigned to placebo, respectively via IV infusion every 7±1 days for 4 doses; followed by 900 mg eculizumab, or placebo, respectively, via IV infusion 7±1 day later; followed by a maintenance dose of 900 mg eculizumab, or placebo, respectively, via IV infusion every 14±2 days for a total of 26 weeks of treatment.

Measures of Clinical Efficacy

[0075] There were two co-primary endpoints in the study: (1) stabilization of hemoglobin levels, defined as a hemoglobin value maintained above the individual hemoglobin set point in the absence of transfusions for the entire 26-week treatment period, and (2) reduction in units of PRBCs transfused during the 26-week treatment phase of the study. The trigger for transfusion during the study period remained unchanged for each patient, as compared with their care before entry into the study: patients received blood transfusions when they had symptoms resulting from anemia and reached their individualized, predetermined "set point". Pre-specified secondary endpoints included transfusion avoidance, hemolysis as measured by LDH area under the curve from baseline to 26 weeks, and QoL changes as measured from baseline to 26 weeks using the Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT-Fatigue) instrument. [13] Pre-specified exploratory analyses included assessment of the EORTC QLQ-C30 instrument, [14] the change in LDH from baseline through week 26, and thrombosis. Other pre-specified measurements included pharmacokinetics, pharmacodynamics, and immunogenicity of eculizumab. Time to first transfusion during the 26-week treatment phase and the proportion of PNH type III blood cells were also assessed.

Safety Assessments

[0076] Treatment-emergent adverse events, clinical laboratory tests (e.g., serum chemical analyses and complete blood counts), electrocardiogram data, and vital sign were assessed. Adverse events were defined using the MedDRA preferred terms and tabulated as incidence rates per treatment group.

Statistical Analysis

[0077] For co-primary endpoints, analyses were performed according to the intention to treat using the data from all patients who were randomized and received study drug; stabilization of hemoglobin levels was analyzed using the Fisher's exact test and total PRBC units transfused were analyzed with the Wilcoxon's rank sum test. For comparison of treatment effect on transfusion avoidance, the Fisher's exact test was used on the incidence and the log rank test was used for time to first transfusion. For LDH area under the curve the Wilcoxon's rank sum test was used.

[0078] Quality of life measure of fatigue was assessed using the scoring guidelines for the FACIT-Fatigue instrument.[15] Assessment of quality of life measures based on the EORTC QLQ-C30 instrument was conducted in accordance with the appropriate scoring guidelines The changes of FACIT-Fatigue and EORTC QLQ-C30 scores from baseline through 26 weeks were analyzed using a mixed model, with baseline as covariate, treatment and time as fixed effects, and patient as a random effect. Changes in LDH levels and PNH type III erythrocytes from baseline through 26 weeks were analyzed using the same mixed model. Two-sided tests were used for all analyses. The adverse events and long-term safety checklist were tabulated separately and compared between treatments using the Fisher's exact test. A p-value ≤0.05 was considered to be statistically significant.

RESULTS

Characteristics of Patients

[0079] A total of 115 PNH patients were screened. Six patients did not meet the inclusion/exclusion criteria during the screening period. Twenty-one other patients did not receive a qualifying transfusion and were not randomized into the

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

[0080] Patient characteristics were similar in the eculizumab- and placebo-treated cohorts: median age, 41 (range 20-85) and 35 (range 18-78) years; median duration of PNH, 4.2 (range 0.8 to 29.7) and 9.2 (range 0.4 to 38.3) years; patients with history of aplastic anemia, 4 and 11; history of myelodysplastic syndrome, 1 and 0; and history of thrombosis, 9 (16 events) and 8 (11 events). Stable usage of concomitant medications at baseline in the eculizumab- and placebo-treated groups included the following: erythropoietin, 3 patients and 0 patients; cyclosporine, 1 and 1; anticoagulants (coumarins or heparins) 21 and 11; and steroids (glucocorticoids or androgenic steroids), 12 and 12, respectively.

[0081] Of the 87 patients randomized, 85 completed the trial. Two patients who did not complete the trial had been randomized to the eculizumab arm: one patient discontinued due to the inconvenience of travel to the study site and the second patient became pregnant. Ten patients in the placebo-treatment group discontinued infusions, in all cases due to perceived lack of efficacy, but they remained in the study for monitoring purposes.

Pharmacokinetics/Pharmacodynamics

[0082] In 42 of the 43 eculizumab-treated patients the levels of drug during the maintenance period (900 mg every 2 weeks±2 days) were sufficient to completely block serum hemolytic activity (mean trough value at week 26 of 101.8 μg/mL). A single patient did not sustain therapeutic trough levels of eculizumab and demonstrated a breakthrough in complement blockade during the last few days of each dosing interval. These breakthroughs were clinically manageable and quickly resolved following the next dose.

Hemolytic Efficacy Variables

[0083] The impact of terminal complement inhibition with eculizumab on chronic intravascular hemolysis in PNH patients was demonstrated in this study by an immediate (one week) and sustained decrease in mean levels of LDH (Figure 1A). The median LDH area under the curve during the 26-week study period was reduced 85.8% in eculizumab- relative to placebo-treated patients (P<0.001). The mean LDH level decreased from 2199.7±157.7 IU/L at baseline to 327.3±67.6 IU/L by 26 weeks in eculizumab-treated patients while levels in placebo-treated patients remained consistently elevated with values of 2259.0±158.5 IU/L at baseline and 2418.9±140.3 IU/L at 26 weeks (P<0.001, for eculizumab versus placebo). A second biochemical measure of hemolysis, serum aspartate aminotransferase (AST), also showed a statistically significant improvement following eculizumab- versus placebo-treatment (data not shown). Haptoglobin levels were statistically significantly increased in eculizumab- as compared to placebo-treated patients but mean levels of haptoglobin were still below normal levels in eculizumab-treated patients (data not shown).

[0084] Figure 1A shows the degree of intravascular hemolysis in PNH patients, demonstrated by mean lactate dehydrogenase (LDH) levels (±standard error) from baseline (study week 0) to week 26 for both eculizumab- and placebo-treated patient populations. Screening occurred up to 3 months prior to study week 0. The upper limit of the normal range (103-223 IU/L) for LDH is indicated by a dashed line. LDH was reduced to a mean level just above the upper limit of normal at week 26 for eculizumab-treated patients; 15 of 41 patients who completed the study demonstrated LDH levels within the normal range. All placebo-treated patients remained at least 5 times above the upper limit of normal at week 26. The P value is based on a mixed model analysis from baseline through week 26. Figure 1B shows the mean proportion (±standard error) of PNH type III erythrocytes assessed for placebo- and eculizumab-treated patients. The screening visit occurred up to 3 months prior to study week 0. The P value is based on a mixed model analysis from baseline through week 26.

[0085] A corollary to the reduction in intravascular hemolysis during eculizumab treatment was an observed increase in the PNH type III erythrocyte population (Figure 1B). The mean proportions of type III erythrocytes increased from 28.1±2.0% at baseline to 56.9±3.6% by week 26 for eculizumab-treated patients while proportions in the placebo group remained constant with mean values of 35.7±2.8% before treatment to 35.5±2.8% at 26 weeks (P<0.001, for eculizumab versus placebo). By contrast, the proportions of PNH type III granulocytes and monocytes did not change significantly between the treatment groups during the treatment period and were greater than 90% at week 26.

CLINICAL EFFICACY

Co-primary endpoints

[0086] The co-primary efficacy endpoints in the TRIUMPH trial were stabilization of hemoglobin levels and reduction in PRBC units transfused. At the end of the treatment period, 48.8% of eculizumab-treated patients had maintained levels of hemoglobin above the pre-specified set-point (median set-point value of 7.7 g/dL for both treatment groups) in

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the absence of transfusions, whereas stabilization of hemoglobin did not occur in any of the patients in the placebo group (P<0.001; Table 1). By week 26, the median of PRBC units transfused per patient was 0 in the eculizumab group and 10.0 in the placebo cohort (P<0.001), while the mean of PRBC units transfused was 3.0 and 11.0 in the eculizumab and placebo cohorts, respectively. In the 6-month period prior to the study, the median of PRBC units transfused per patient was 9.0 in the eculizumab cohort and 8.5 in placebo patients while the mean of PRBC units transfused was 9.6±0.6 and 9.7±0.7, respectively. Mean hemoglobin levels were similar between the treatment groups at baseline (10.0±1.8 g/dL in eculizumab-treated patients and 9.7±1.8 g/dL in placebo-treated patients) and did not substantially change by week 26 (10.1±2.5 g/dL and 8.9±2.2 g/dL in eculizumab and placebo cohorts, respectively).

[0087] The median time to first transfusion was not reached during the study period in eculizumab-treated patients (it was greater than 26 weeks) while the placebo group reached the median time to first transfusion in only 4 weeks (P<0.001; Figure 2). Transfusion avoidance was achieved in 51.2% and 0% of the eculizumab and placebo cohorts, respectively (P<0.001). By the end of the 26-week treatment period, the total PRBC units transfused were 131 in eculizumab-treated patients versus 482 in the placebo group (Table 1). By contrast, in the 6-month period prior to the study, total PRBC units transfused in the eculizumab- and placebo-cohorts were 413 and 417, respectively.

Table 1

Stabilization of Hemoglobin Levels and Reduction in Transfusion Requirements during Eculizumab Treatment					
	PRE-TREATMENT HISTORY*		TREATMENT PERIOD		
	Placebo	Eculizumab	Placebo	Eculizumab	P Value
†Stabilization of Hemoglobin Levels in the absence of transfusions (percent of patients)	NA	NA	0	48.8	<0.001‡
†Units Transfused per Patient					
Median	8.5	9.0	10	0	<0.001§
Mean±SE	9.7±0.7	9.6±0.6	11.0±0.83	3.0±0.67	
Total Units Transfused per Group	417	413	482	131	
*Twelve month historical transfusion data normalized to 6 months †Co-primary endpoints ‡Base on 2-sided Fisher's exact test §Based on Wilcoxon's rank sum test NA, not applicable					

Improvements in quality of life measures

[0088] Assessments of quality of life in PNH patients during eculizumab treatment were performed using two different instruments; FACIT-Fatigue and the EORTC QLQ-C30. Eculizumab-treated patients showed a mean increase (improvement) in the FACIT-Fatigue score of 6.4±1.2 points from baseline to 26-weeks while the mean score in placebo patients decreased by 4.0±1.7 points, a total difference between the treatment groups of 10.4 points (Figure 3). Mixed model analysis of covariance demonstrated a statistically significant difference between treatment groups (P<0.001).

[0089] For the EORTC instrument, improvements were observed with eculizumab-treatment in each subscale. Statistically significant improvements with eculizumab-compared with placebo-treated groups were observed in the following quality of life subscales (Table 2): global health status (P<0.001), physical functioning (P<0.001), emotional functioning (P=0.008), cognitive functioning (P=0.002), role functioning (P<0.001), social functioning (P=0.003), fatigue (P<0.001), pain (P=0.002), dyspnea (P<0.001), appetite loss (P<0.001), and insomnia (P=0.014). The improvements with eculizumab treatment in the other scales, including nausea and vomiting, diarrhea, constipation, and financial difficulties, did not reach statistical significance.

Table 2

Effect of Eculizumab Treatment on Quality of Life Assessed by the EORTC QLQ-C30 Instrument				
	Mean Change from Baseline to Week 26*			
	Placebo	**Eculizumab**	**Absolute Difference**	**P Value†**
Global Health Status	-8.5	10.9	19.4	<0.001

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(continued)

Effect of Eculizumab Treatment on Quality of Life Assessed by the EORTC QLQ-C30 Instrument Mean Change from Baseline to Week 26*				
	Placebo	Eculizumab	Absolute Difference	P Value†
Functional				
Role	-6.9	17.9	24.8	<0.001
Social	2.0	16.7	14.6	=0.003
Cognitive	-6.1	7.9	14.0	=0.002
Physical	-3.5	9.4	13.0	<0.001
Emotional	-3.7	7.5	11.2	=0.008
Symptoms/Single Items				
Fatigue	10.0	-16.9	27.0	<0.001
Pain	5.3	-12.3	17.6	=0.002
Dyspnea	8.9	-7.9	16.9	<0.001
Appetite Loss	3.3	-10.3	13.6	<0.001
Insomnia	4.9	-7.9	12.8	=0.014
Financial Difficulties	0.0	-10.3	10.3	=0.186
Constipation	0.0	-6.3	6.3	=0.199
Nausea/vomiting	2.8	-0.4	3.2	=0.056
Diarrhea	5.7	4.8	0.9	=0.147

*A positive change indicates an improvement in global health status and functional scales and a negative change indicates an improvement in symptom and single item scales.
†Based on a mixed analysis-of covariance model with visit as a fixed effect, patient as a random effect and baseline as a covariate.

Relationship between FACIT-Fatigue quality of life and intravascular hemolysis

[0090] In order to determine if there was a treatment independent relationship between the FACIT-Fatigue quality of life instrument and intravascular hemolysis, an analysis was performed whereby the mean LDH level (through the 26 week study period) for each TRIUMPH patient was analyzed as a function of the patient's respective mean change in FACIT-Fatigue score from baseline (through the 26 week study period) (see Table 3). For this analysis, mean levels of LDH were divided into 4 groups that included: normal levels, 1-2 times the upper limit of normal (ULN), 2-10 times the upper limit of normal, and greater than 10 times the upper limit of normal. The analysis demonstrated that patients who maintained normal LDH levels throughout the study experienced significant improvements in fatigue when compared to patients who had incrementally higher levels of LDH throughout the study (p=0.0048). These data establish a clear link between increasing intravascular hemolysis as measured by LDH levels and decreased quality of life as measured by the FACIT-Fatigue quality of life instrument.

Table 3

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

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Safety

[0091] There were no deaths in the study. Serious adverse events (SAEs) were reported for 13 patients, of which 4 occurred in the eculizumab-treated cohort and 9 were in the placebo-treated cohort (see Table 4). All patients recovered without sequelae.

[0092] The most common AEs reported for eculizumab-treated patients were headache, nasopharyngitis, back pain, and upper respiratory tract infection. Headache and back pain occurred more commonly in the eculizumab-treatment group compared with the placebo group. However, the increase in headaches was limited to the first 2 weeks of therapy and was mild to moderate. There were no statistically significant differences in incidents rates between treatment groups for any AEs reported.

[0093] One episode of thrombosis (Budd-Chiari) occurred in a placebo-treated patient. There were no thromboses in eculizumab-treated patients.

[0094] Only one patient showed a detectable level of anti-eculizumab antibodies in the eculizumab-treated cohort; this response was weak (did not titrate), occurred at only one time point and did not result in a disruption of complement blockade.

Table 4
Adverse Event Reporting

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

*By preferred terms
†Occurring in 10% or more of patients
‡Sixteen of 19 patients experienced headache within 48 hours of infusion
§Following the first 2 weeks of dosing, 20.9% of eculizumab- and 22.7% of placebo-treated patients experienced headache

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DISCUSSION

[0095] Chronic intravascular hemolysis with periods of acute exacerbation are the classical manifestations of PNH, frequently resulting in anemia, the need for transfusions to sustain hemoglobin levels, and deterioration in quality of life. In the phase III pivotal TRIUMPH study, we examined the effect of terminal complement inhibition with eculizumab on hemoglobin levels and transfusion requirements in patients with PNH. Forty-nine percent of patients treated with eculizumab over the 6-month period demonstrated stabilization of hemoglobin in the absence of transfusions compared to no patients in the placebo arm of the trial. Over 50% of eculizumab-treated patients were transfusion independent during the entire study compared to none in the placebo arm, and the overall mean transfusion rate was reduced by 73%. Moreover, even in patients who did not achieve transfusion independence, eculizumab treatment was associated with a 44% reduction in the rate of transfusion (data now shown).

[0096] Lactate dehydrogenase, a biochemical marker of hemolysis in PNH,[9] was immediately and consistently decreased in all eculizumab-treated patients, while patients in the placebo cohort continued to hemolyze with levels of LDH exceeding 5 times the upper limit of the normal range in all patients at the study end. Levels of LDH were reduced into the normal range in approximately one-third of eculizumab-treated patients, while the remainder stabilized at a level just above the upper limit of normal suggesting residual low level hemolysis in some patients. Levels of haptoglobin, a more sensitive marker of the presence of cell free hemoglobin in the circulation, were undetectable in most patients. Low level hemolysis in a subset of eculizumab-treated patients is possibly due to an inherent decrease in survival of these cells or C3b-mediated, extravascular clearance of PNH erythrocytes through the reticuloendothelial system.[17]

[0097] Before eculizumab treatment, hemoglobin levels in study patients were artificially maintained by frequent transfusion. Therefore, stabilization of hemoglobin levels with a concomitant cessation of or reduction in transfusions represents a net increase in endogenous hemoglobin levels. Our data suggest that resolution of hemolysis with eculizumab results in a new steady state hemoglobin level determined by a balance between the extent of the underlying bone marrow dysfunction, the number of PNH erythrocytes that are preserved by eculizumab therapy and the new level (if any) of transfusion requirement.

[0098] Patients with PNH generally experience markedly impaired quality of life characterized by fatigue, anemia, thrombosis, and pulmonary hypertension as well as smooth muscle dystonia including abdominal pain, dysphagia, and erectile dysfunction.[9,10,18] These symptoms have been attributed to both excessive intravascular hemolysis and downstream scavenging of nitric oxide by cell free hemoglobin in plasma. The reduction of intravascular hemolysis in eculizumab-treated patients in the current study was associated with significant improvements in the fatigue component of quality of life relative to placebo-treated patients as assessed via the FACIT-Fatigue instrument. Further, eculizumab therapy was associated with a median increase of 6.4 points over baseline values established before treatment. It has previously been demonstrated that an increase of 3 or more points from baseline represents a clinically important difference in this quality of life instrument.[19] Patients who received eculizumab also experienced a significant improvement in most domains of the EORTC QLQ-30 relative to the placebo-treated cohort including global health status, physical functioning, emotional functioning, cognitive functioning, role functioning, social functioning, fatigue, pain, dyspnea, appetite loss, and insomnia. Improvement in the fatigue component of the EORTC QLQ-30 provides support for the improvement demonstrated in the FACIT-Fatigue instrument during eculizumab therapy. Importantly, these improvements in quality of life in the eculizumab-treated patients occurred despite similar levels of erythrocyte hemoglobin in the two treatment groups, further supporting the contribution of hemolysis per se, as opposed to anemia, in mediating the poor quality of life in PNH patients. Clinical assessment of additional life quality-related symptoms of PNH, including abdominal pain, dysphagia, and erectile dysfunction, have also been reported to improve during eculizumab therapy.[20]

[0099] Eculizumab was safe and well-tolerated. There were no deaths in the study and only a single thrombotic event which occurred in a placebo patient in a site (the hepatic veins) which is typical of the thrombosis in PNH. The relative brief duration of this study was not sufficient to address the relevant issue of a possible protection from thrombosis by terminal complement inhibition with eculizumab.

[0100] Adverse events were generally mild with headache occurring at increased frequency in the eculizumab-treated patients; however, this increased frequency did not persist following the first two doses of therapy. There were 4 SAEs in the eculizumab treatment group and 9 SAEs in the placebo group. There was no evidence of increased infection risk in eculizumab-treated patients during the study period. One eculizumab-treated patient showed a low level of anti-eculizumab antibodies at one time point during the study which did not persist and did not result in a disruption of complement blockade. There were no AEs associated with eculizumab withdrawal in the 2 eculizumab-treated patients who did not complete the trial. Additional safety assessments, as well as efficacy measures, are being examined in an ongoing multi-center, open-label Phase III safety trial of eculizumab (SHEPHERD) in approximately 95 patients with PNH.

[0101] Results from the current randomized, double-blind, placebo-controlled, global study show that terminal complement inhibition with eculizumab appears to be a safe and effective therapy for patients with the rare disorder PNH. Treatment with eculizumab reduced intravascular hemolysis, and stabilized hemoglobin levels despite a reduction of transfusions, to the point where most PNH patients were rendered transfusion independent. Substantial and clinically

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meaningful improvements in fatigue and other key quality of life parameters were also demonstrated. All of the 85 patients who completed the study elected to receive eculizumab in an open-label extension study and all currently remain on drug. The results of the TRIUMPH study indicate that terminal complement inhibition with eculizumab safely and effectively addresses an important consequence of the underlying genetic defect in PNH hematopoietic stem cells by providing a therapeutic replacement for the terminal complement inhibitor deficiency.

[0102] The present invention provides among other things treatment with an inhibitor of complement. Many variations of the invention will become apparent to those skilled in the art upon review of this specification. The full scope of the invention should be determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.

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Ref Type: Abstract

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22. Hill A, Wang X, Sapsford RJ et al. Nitric oxide Consumption and Pulmonary Hypertension in Patients with Paroxysmal Nocturnal Hemoglobinuria. Blood 2005;106:A1046.

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[0103]

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SEQ ID NO: 8 - Eculizumab CDRL1
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Lys Asp Arg Val Thr Met Thr Arg Asp Thr Ser Thr Ser Thr Val Tyr
65                  70                  75                  80

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Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp
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Claims

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

Patentansprüche

1. Pharmazeutische Zusammensetzung zur Verwendung in der Behandlung eines Patienten, der an paroxysmaler nächtlicher Hämoglobinurie (PNH) leidet, wobei die Zusammensetzung eine 300-mg-Eculizumab-Einmal-Darreichungsform ist, umfassend 30 ml einer 10 mg Eculizumab pro ml enthaltenden sterilen, konservierungsmittelfreien Lösung.

Revendications

1. Composition pharmaceutique pour une utilisation dans le traitement d'un patient atteint d'une hémoglobinurie paroxystique nocturne (HPN), dans laquelle la composition est une forme galénique à usage unique de 300 mg d'eculizumab comprenant 30 ml d'une solution à 10 mg d'eculizumab/ml, stérile et sans conservateur.

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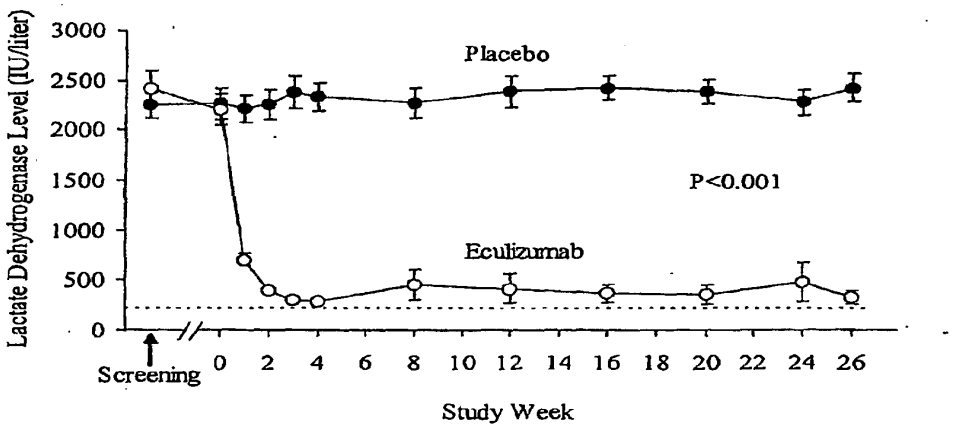


FIGURE 1A

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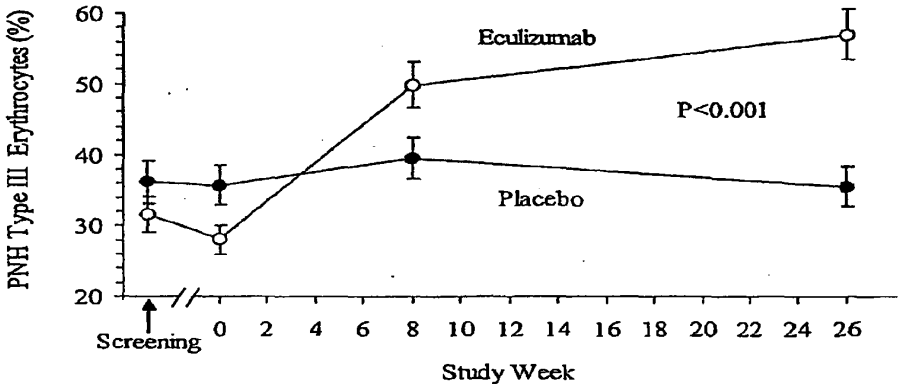


FIGURE 1B

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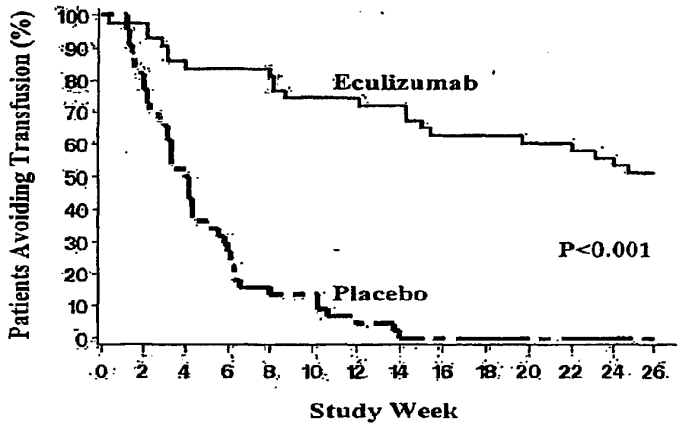


FIGURE 2

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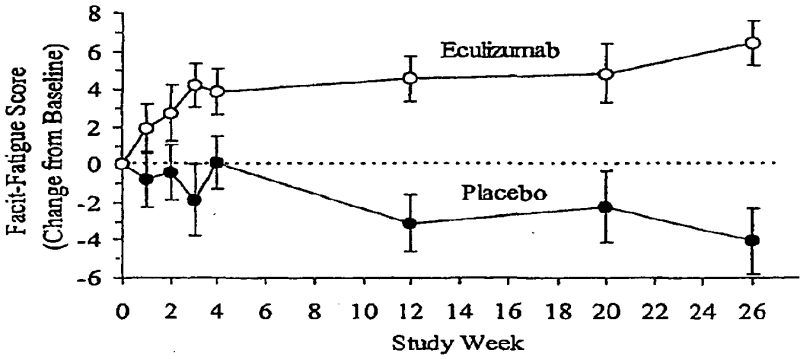


FIGURE 3

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REFERENCES CITED IN THE DESCRIPTION

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

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

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TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

BACKGROUND

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

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

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

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SUMMARY

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

BRIEF DESCRIPTION OF THE FIGURES

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

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

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

DETAILED DESCRIPTION

I. Definitions

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

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

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

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

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

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

II. Overview

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

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

III The Complement System

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

IV Measures of Quality of Life

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

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

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

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

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

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

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

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

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

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

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

V Inhibitors of the Complement Cascade

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

VI Methods of Treatment

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

VII Pharmaceutical Formulations and Uses

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

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

In certain embodiments, the pharmaceutical composition is in a single unit dosage form. In certain embodiments, the single unit dosage form is a 300 mg unit dosage form. In certain embodiments, the pharmaceutical composition is lyophilized. In certain embodiments, the pharmaceutical composition is a sterile solution. In certain embodiments, the pharmaceutical composition is a preservative free formulation. In certain embodiments, the pharmaceutical composition comprises a 300 mg single-use formulation of 30 ml of a 10 mg/ml sterile, preservative free solution. In certain embodiments, the antibody is administered according to the following protocol:

600 mg via 25 to 45 minute IV infusion every 7 ± 2 days for the first 4 weeks, followed by 900 mg for the fifth dose 7 ± 2 days later, then 900 mg every 14 ± 2 days thereafter. Antibody is administered via IV infusion over 25 to 45 minute.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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EXEMPLIFICATION

METHODS

Patient Selection

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

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

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

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

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

Study Design

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

Measures of Clinical Efficacy

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

Safety Assessments

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

Statistical Analysis

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

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

RESULTS

Characteristics of Patients

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

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

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

Pharmacokinetics/Pharmacodynamics

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

Hemolytic Efficacy Variables

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

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

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

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

CLINICAL EFFICACY

Co-primary endpoints

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

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

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

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Table 1

Stabilization of Hemoglobin Levels and Reduction

in Transfusion Requirements during Eculizumab Treatment

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

*Twelve month historical transfusion data normalized to 6 months

†Co-primary endpoints

‡Base on 2-sided Fisher's exact test

§Based on Wilcoxon's rank sum test

NA, not applicable

Improvements in quality of life measures

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

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

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

Table 2

Effect of Eculizumab Treatment on

Quality of Life Assessed by the EORTC QLQ-C30 Instrument

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

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

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

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

Relationship between FACIT-Fatigue quality of life and intravascular hemolysis

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

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Table 3

Relationship between FACIT-Fatigue and Intravascular Hemolysis

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

Safety

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

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

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

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

Table 4

Adverse Event Reporting

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

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Viral infection	5 (11.4)	1 (2.3)

*By preferred terms

†Occurring in 10% or more of patients

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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SEQUENCES

SEQ ID NO: 1 – Eculizumab V_H

QVQLVQSGAEVKKPGASVKVSCKASGYIFSNYWIQWVRQAPGQGLEWMGEILPG
SGSTEYTENFKDRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARYFFGSSPNWYF
DVWGQGTLVTVSSA

SEQ ID NO: 2 – Eculizumab Heavy chain

QVQLVQSGAEVKKPGASVKVSCKASGYIFSNYWIQWVRQAPGQGLEWMGEILPG
SGSTEYTENFKDRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARYFFGSSPNWYF
DVWGQGTLVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVE
RKCCVECPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFN
WYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLP
SSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQ
PENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSL
SLSLGK

SEQ ID NO: 3 – Eculizumab V_L

MDMRVPAQLLGLLLLWLRGARCDIQMTQSPSSLSASVGDRVTITCGASENIYGAL
NWYQQKPGKAPKLLIYGATNLADGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQ
NVLNTPLTFGQGTKVEIKRT

SEQ ID NO: 4 – Eculizumab Light chain

MDMRVPAQLLGLLLLWLRGARCDIQMTQSPSSLSASVGDRVTITCGASENIYGAL
NWYQQKPGKAPKLLIYGATNLADGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQ
NVLNTPLTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKV
QWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG
LSSPVTKSFNRGEC

SEQ ID NO: 5 – Eculizumab CDRH1

NYWIQ

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SEQ ID NO: 6 – Eculizumab CDRH2
EILPGSGSTEYTENFKD

SEQ ID NO: 7 – Eculizumab CDRH3
YFFGSSPNWYFDV

SEQ ID NO: 8 – Eculizumab CDRL1
GASENIYGALN

SEQ ID NO: 9 – Eculizumab CDRL2
GATNLAD

SEQ ID NO: 10 – Eculizumab CDRL3
QNVLNTPLT

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WHAT IS CLAIMED IS:

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

52. The method of claim 50 wherein the FACIT-Fatigue score increases by ≥ 4 points.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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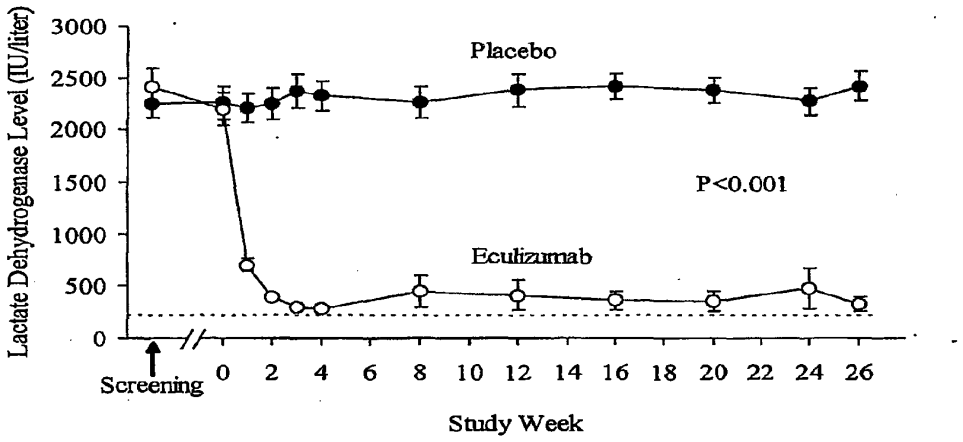


FIGURE 1A

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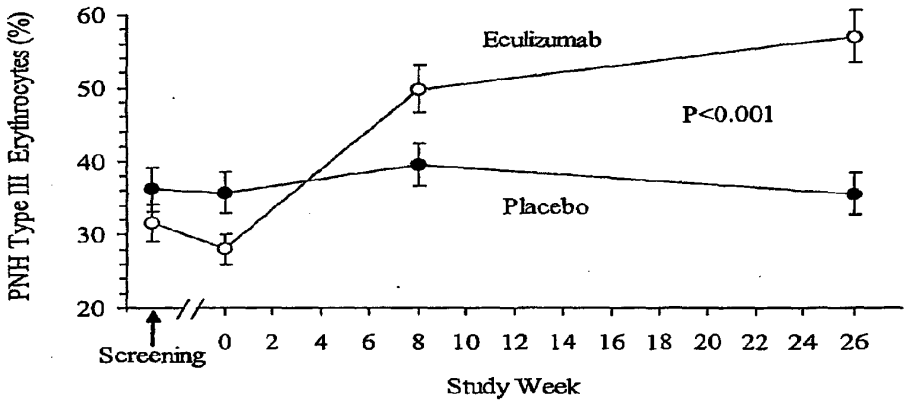


FIGURE 1B

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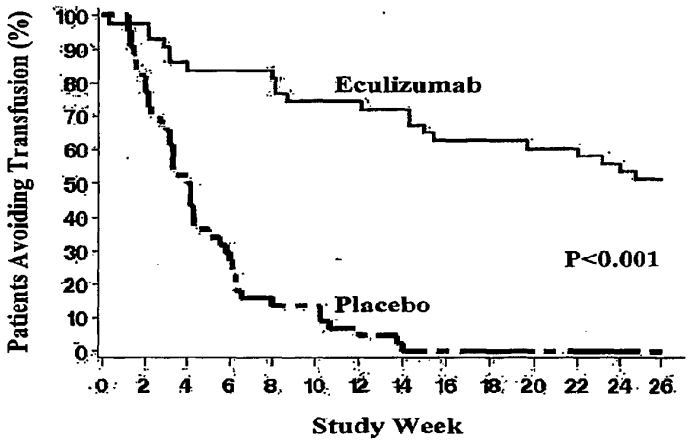


FIGURE 2

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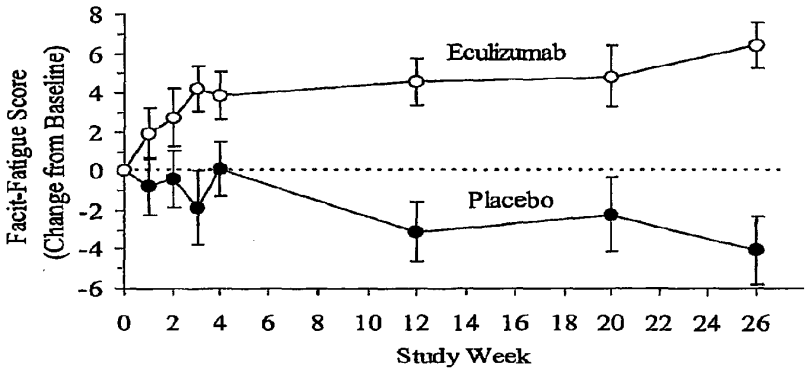


FIGURE 3

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2007/006606

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/7105 A61K38/00 A61K39/395 A61P7/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2005/191298 A1 (BELL LEONARD [US] ET AL) 1 September 2005 (2005-09-01) the whole document	1-90
A	US 2005/221382 A1 (ROTHER RUSSELL P [US]) 6 October 2005 (2005-10-06) page 5, left-hand column, paragraph 40	1-90

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 July 2007	27/07/2007

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Collura, Alessandra

Form PCT/ISA/210 (second sheet) (April 2005)

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2007/006606

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1-90
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-90 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (January 2004)

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Page 5755

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2007/006606

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2005191298 A1	01-09-2005	AU 2005209854 A1	18-08-2005
		CA 2554632 A1	18-08-2005
		CN 1938047 A	28-03-2007
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		US 2005169921 A1	04-08-2005
		WO 2005074607 A2	18-08-2005
US 2005221382 A1	06-10-2005	NONE	

Form PCT/ISA/210 (patent family annex) (April 2005)

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US 20030202972A1

(19) **United States**

(12) **Patent Application Publication** (10) **Pub. No.: US 2003/0202972 A1**

Andya et al. (43) **Pub. Date: Oct. 30, 2003**

(54) **PROTEIN FORMULATION**

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(73) Assignee: **Genentech, Inc.**

(21) Appl. No.: **10/428,728**

(22) Filed: **May 2, 2003**

Related U.S. Application Data

(60) Division of application No. 09/809,511, filed on Mar. 14, 2001, which is a continuation of application No. 08/615,369, filed on Mar. 14, 1996, now Pat. No. 6,267,958.

(60) Provisional application No. 60/029,182, filed on Jul. 27, 1995, now abandoned.

Publication Classification

(51) **Int. Cl.**[7] **A61K 39/395**; A61K 31/7012

(52) **U.S. Cl.** **424/131.1**; 424/144.1; 514/53

(57) **ABSTRACT**

A stable lyophilized protein formulation is described which can be reconstituted with a suitable diluent to generate a high protein concentration reconstituted formulation which is suitable for subcutaneous administration. For example, anti-IgE and anti-HER2 antibody formulations have been prepared by lyophilizing these antibodies in the presence of a lyoprotectant. The lyophilized mixture thus formed is reconstituted to a high protein concentration without apparent loss of stability of the protein.

Exhibit no. D9
of the letter / report / expert opinion / plaint / defense
dated 09.08.2017
HOFFMANN · EITLE
Patent- und Rechtsanwälte
81925 München, Arabellastr. 30

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Page 5757

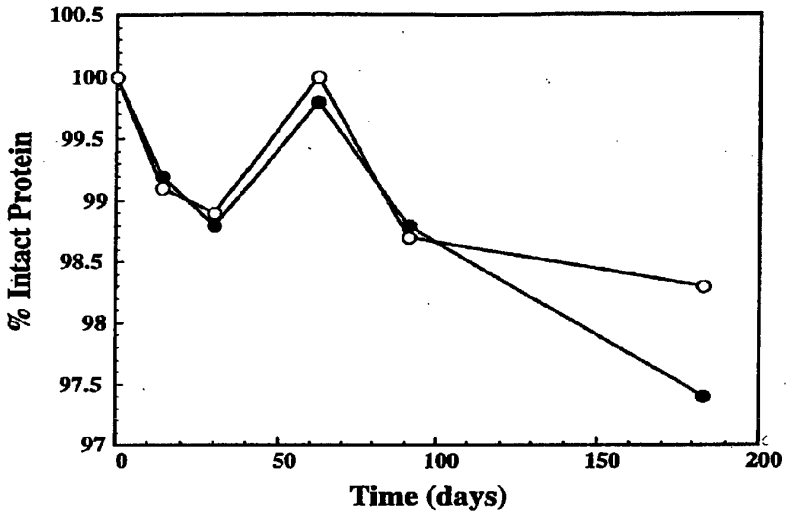


FIG. 1

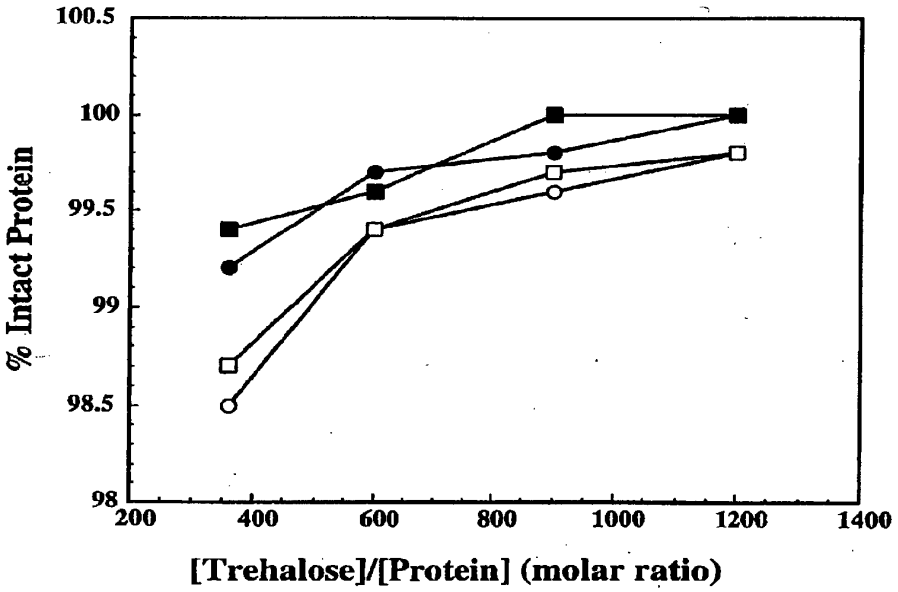


FIG. 2

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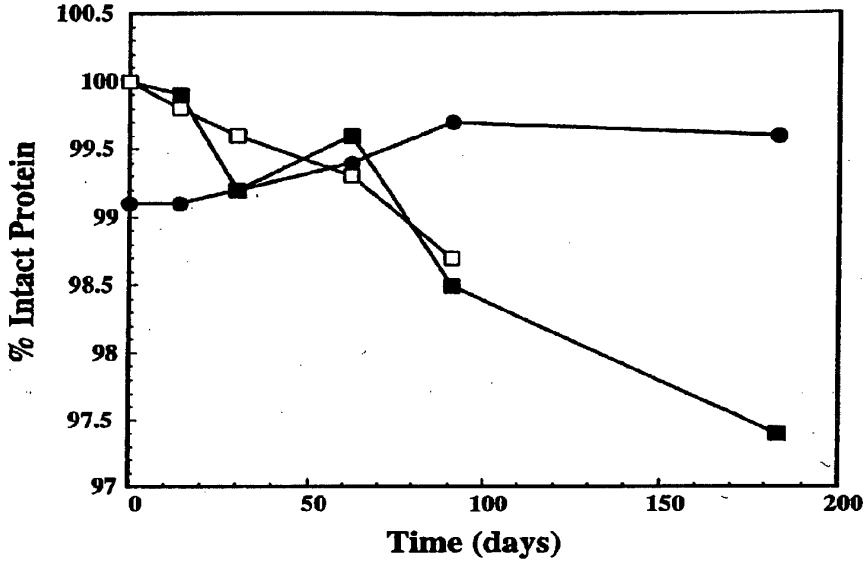


FIG. 3

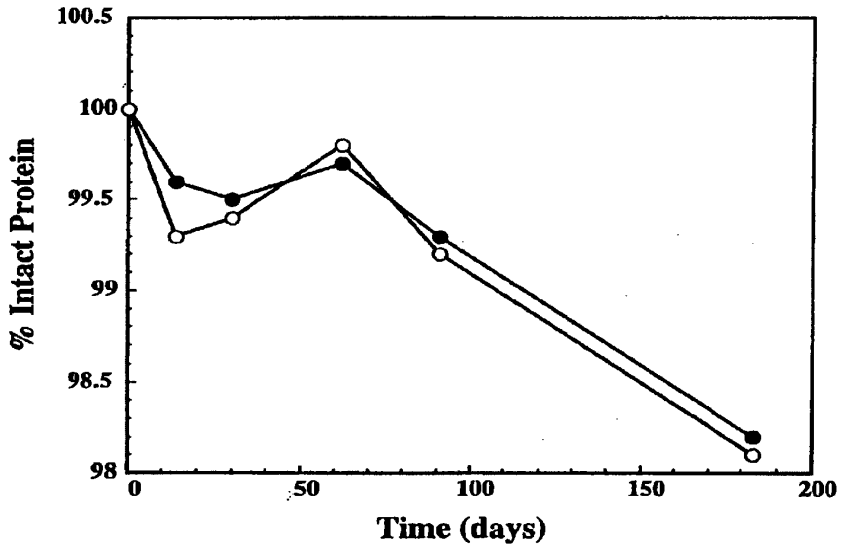


FIG. 4

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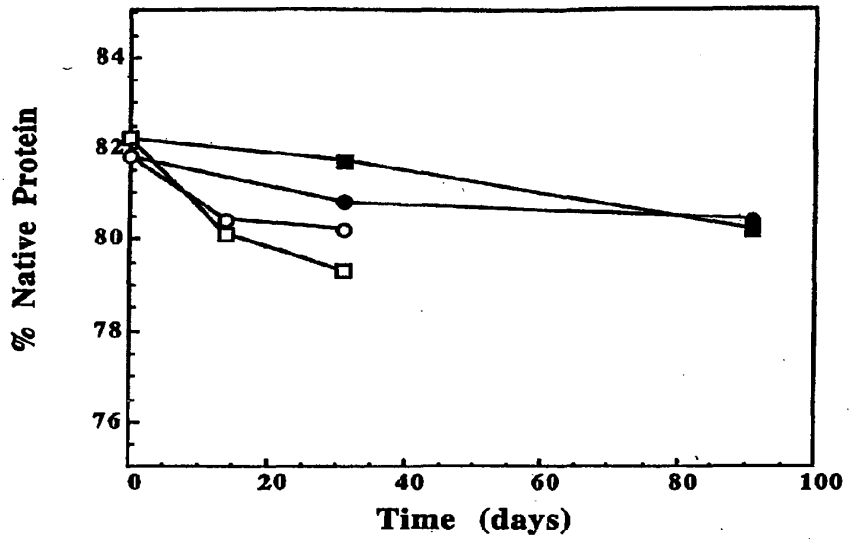


FIG. 5

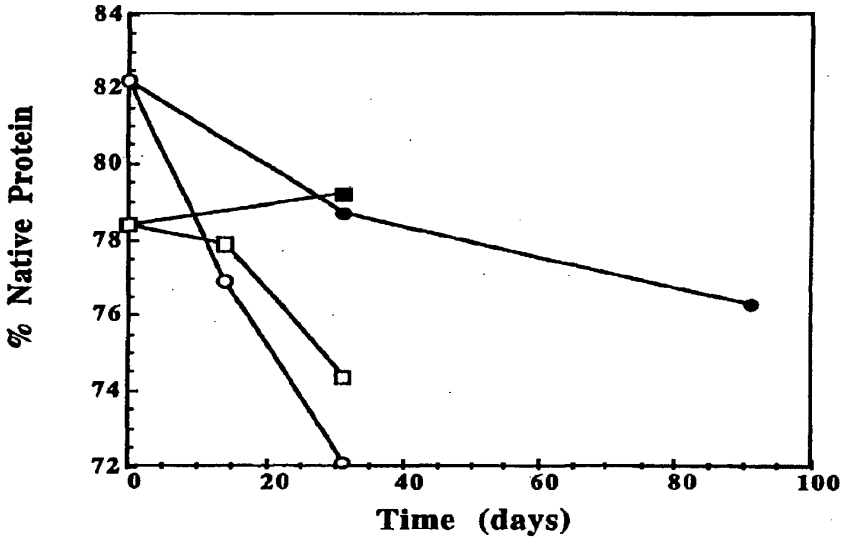


FIG. 6

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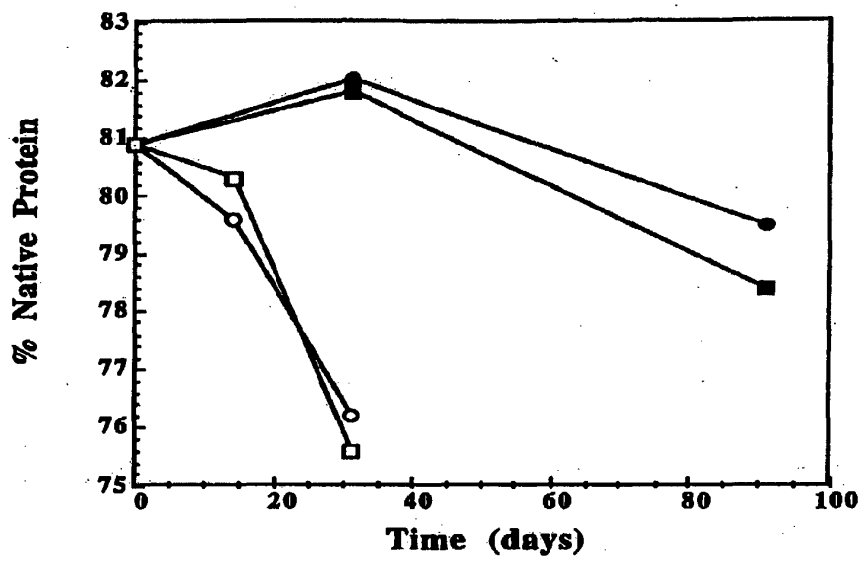


FIG. 7

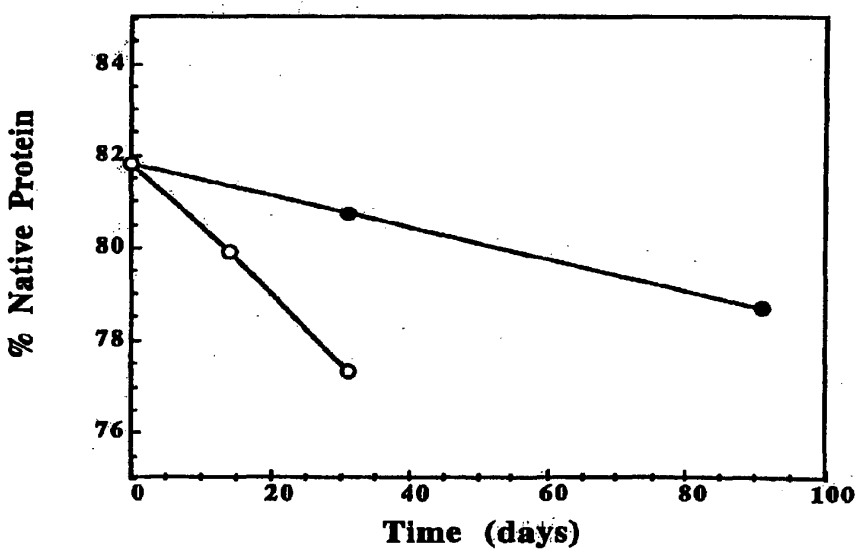


FIG. 8

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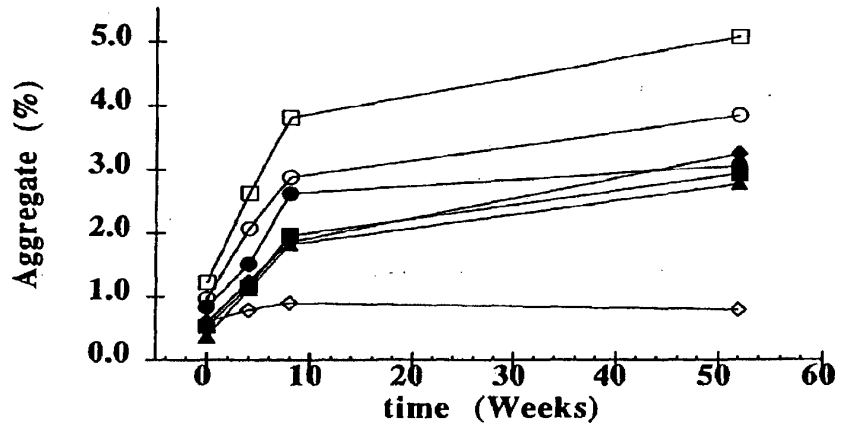


FIG. 9

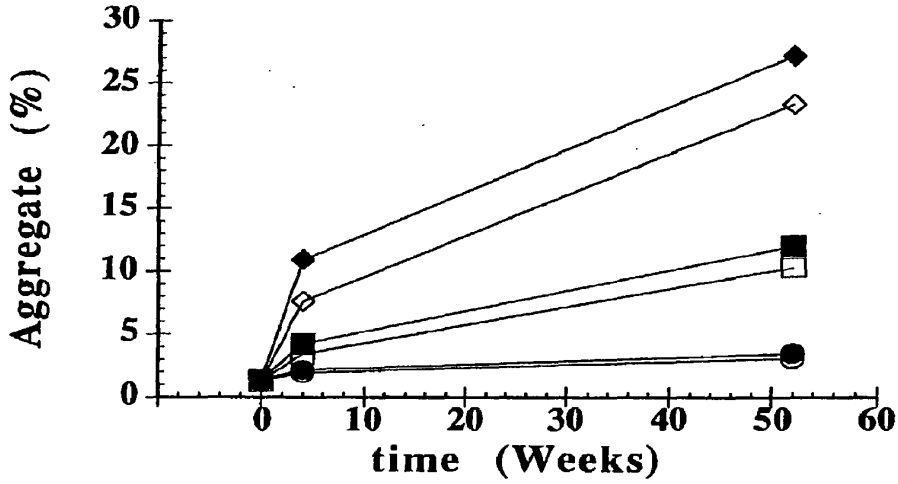


FIG. 10

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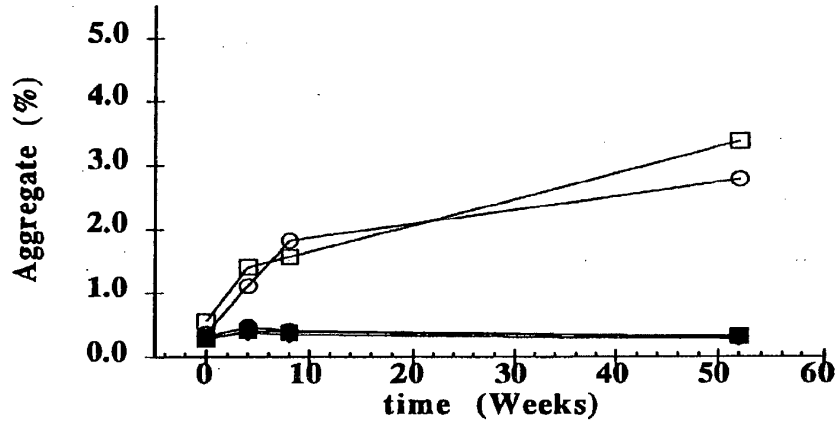


FIG. 11

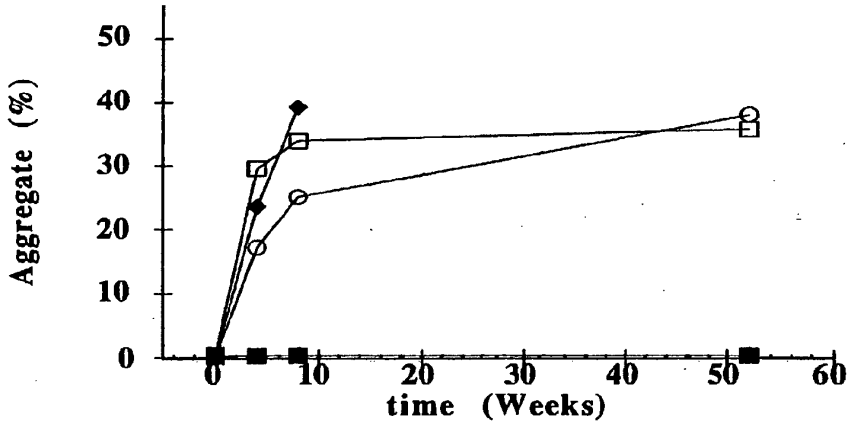


FIG. 12

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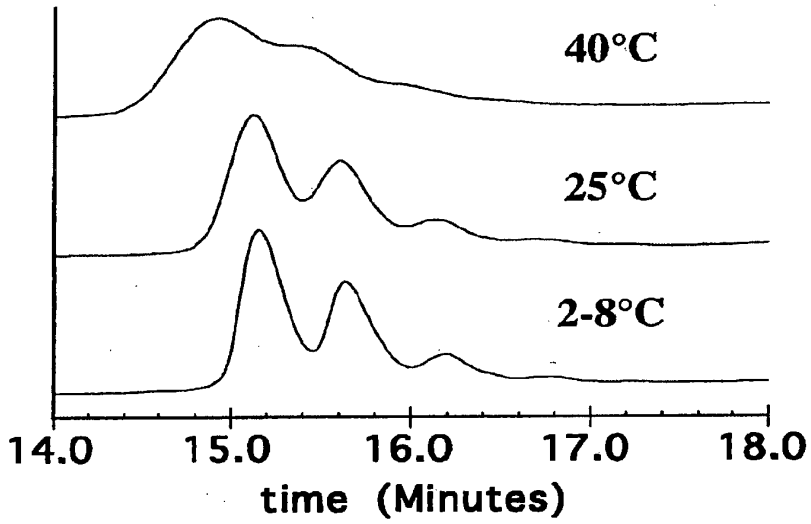


FIG. 13

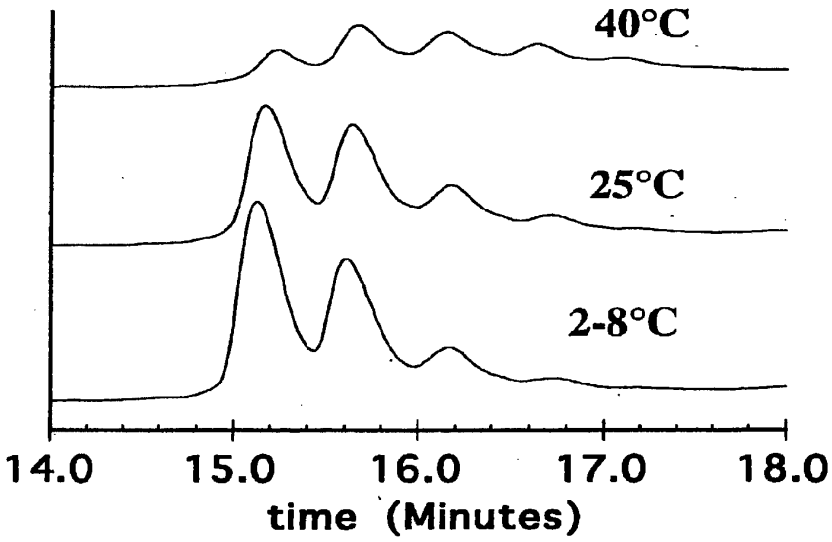


FIG. 14

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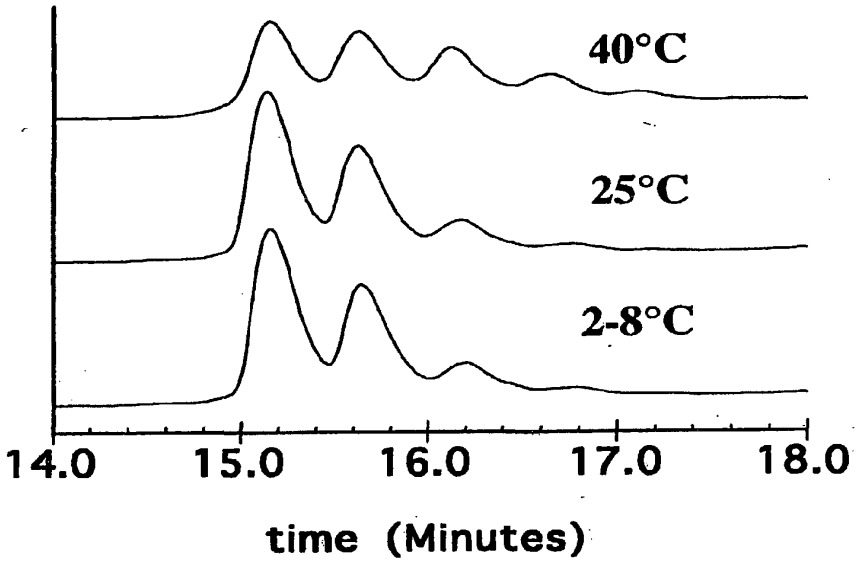


FIG. 15

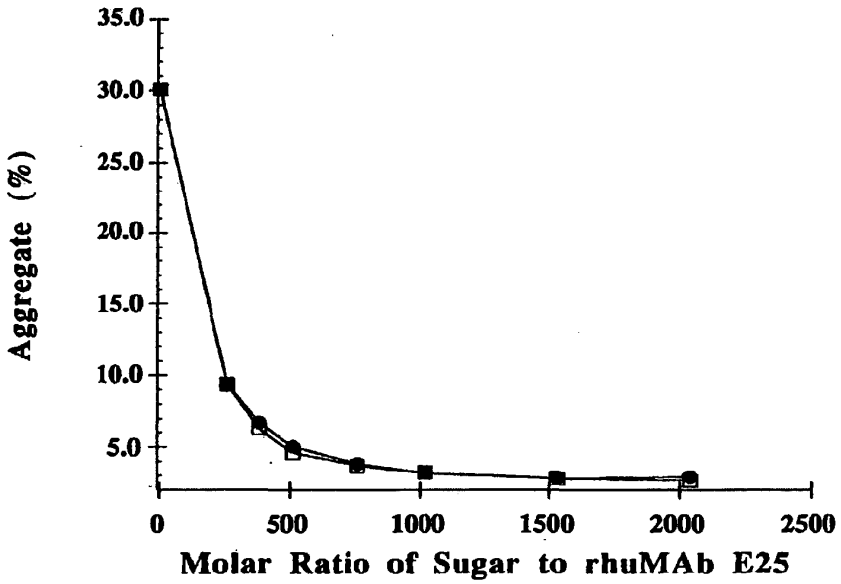


FIG. 16

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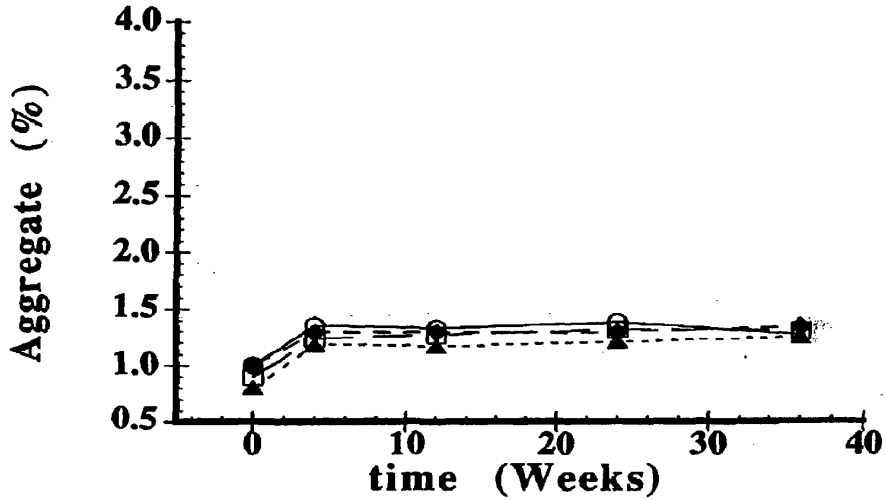


FIG. 17

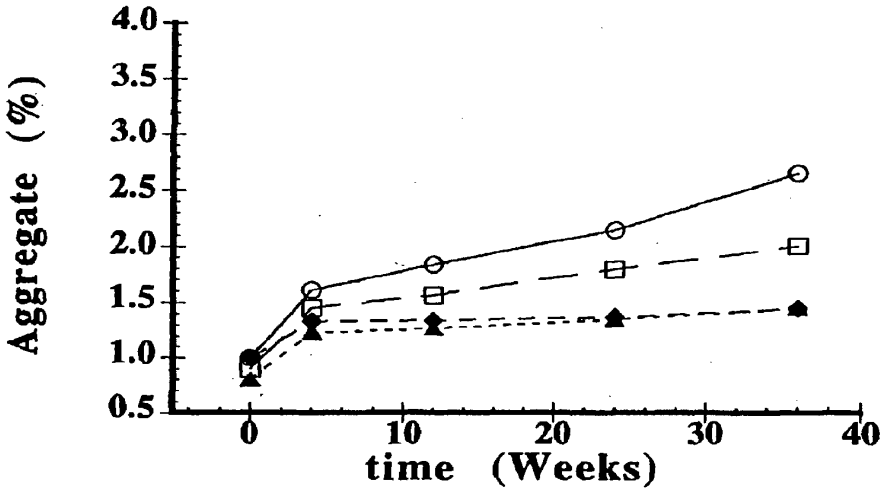


FIG. 18

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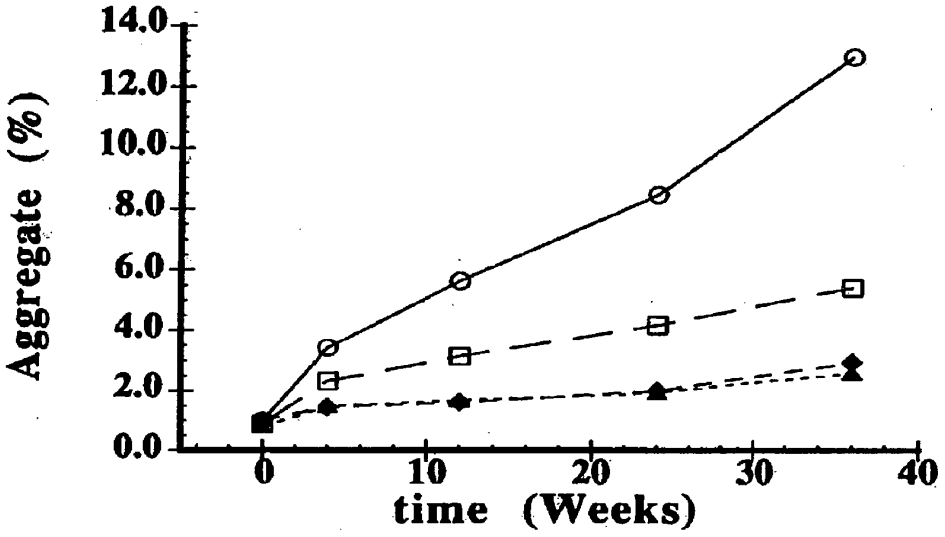


FIG. 19

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PROTEIN FORMULATION

[0001] This is a divisional of U.S. Ser. No. 09/809,511, filed Mar. 14, 2001, which is a continuation of U.S. Ser. No. 08/615,369 filed Mar. 14, 1996 (now U.S. Pat. No. 6,267,958 issued Jul. 31, 2001) which is a non-provisional filed under 37 CFR 1.53(b) claiming priority under 35 USC 19(e) to provisional application 60/029,182 filed Jul. 27, 1995, both incorporated herein by reference.

BACKGROUND OF THE INVENTION

[0002] 1. Field of the Invention

[0003] This invention is directed to a lyophilized protein formulation. In particular, it relates to a stable lyophilized protein formulation which can be reconstituted with a diluent to generate a stable reconstituted formulation suitable for subcutaneous administration.

[0004] 2. Description of Related Disclosures

[0005] In the past ten years, advances in biotechnology have made it possible to produce a variety of proteins for pharmaceutical applications using recombinant DNA techniques. Because proteins are larger and more complex than traditional organic and inorganic drugs (i.e. possessing multiple functional groups in addition to complex three-dimensional structures), the formulation of such proteins poses special problems. For a protein to remain biologically active, a formulation must preserve intact the conformational integrity of at least a core sequence of the protein's amino acids while at the same time protecting the protein's multiple functional groups from degradation. Degradation pathways for proteins can involve chemical instability (i.e. any process which involves modification of the protein by bond formation or cleavage resulting in a new chemical entity) or physical instability (i.e. changes in the higher order structure of the protein). Chemical instability can result from deamidation, racemization, hydrolysis, oxidation, beta elimination or disulfide exchange. Physical instability can result from denaturation, aggregation, precipitation or adsorption, for example. The three most common protein degradation pathways are protein aggregation, deamidation and oxidation. Cleland et al. *Critical Reviews in Therapeutic Drug Carrier Systems* 10(4): 307-377 (1993).

[0006] Freeze-drying is a commonly employed technique for preserving proteins which serves to remove water from the protein preparation of interest. Freeze-drying, or lyophilization, is a process by which the material to be dried is first frozen and then the ice or frozen solvent is removed by sublimation in a vacuum environment. An excipient may be included in pre-lyophilized formulations to enhance stability during the freeze-drying process and/or to improve stability of the lyophilized product upon storage. Pikal, M. *Biopharm.* 3(9)26-30 (1990) and Arakawa et al. *Pharm. Res.* 8(3):285-291 (1991).

[0007] It is an object of the present invention to provide a lyophilized protein formulation which is stable upon storage and delivery. It is a further object to provide a stable reconstituted protein formulation which is suitable for subcutaneous administration. In certain embodiments, it is an object to provide a multi-use formulation which is stable for at least the time over which it will be administered to a patient.

SUMMARY OF THE INVENTION

[0008] This invention is based on the discovery that a stable lyophilized protein formulation can be prepared using a lyoprotectant (preferably a sugar such as sucrose or trehalose), which lyophilized formulation can be reconstituted to generate a stable reconstituted formulation having a protein concentration which is significantly higher (e.g. from about 2-40 times higher, preferably 3-10 times higher and most preferably 3-6 times higher) than the protein concentration in the pre-lyophilized formulation. In particular, while the protein concentration in the pre-lyophilized formulation may be 5 mg/mL or less, the protein concentration in the reconstituted formulation is generally 50 mg/mL or more. Such high protein concentrations in the reconstituted formulation are considered to be particularly useful where the formulation is intended for subcutaneous administration. Despite the very high protein concentration in the reconstituted formulation, it has been found that the reconstituted formulation is stable (i.e. fails to display significant or unacceptable levels of chemical or physical instability of the protein) at 2-8° C. for at least about 30 days. In certain embodiments, the reconstituted formulation is isotonic. In spite of the use of lower concentrations of the lyoprotectant to achieve such isotonic formulations upon reconstitution, it was discovered herein that the protein in the lyophilized formulation essentially retains its physical and chemical stability and integrity upon lyophilization and storage.

[0009] When reconstituted with a diluent comprising a preservative (such as bacteriostatic water for injection, BWFI), the reconstituted formulation may be used as a multi-use formulation. Such a formulation is useful, for example, where the patient requires frequent subcutaneous administrations of the protein to treat a chronic medical condition. The advantage of a multi-use formulation is that it facilitates ease of use for the patient, reduces waste by allowing complete use of vial contents, and results in a significant cost savings for the manufacturer since several doses are packaged in a single vial (lower filling and shipping costs).

[0010] Based on the observations described herein, in one aspect the invention provides a stable isotonic reconstituted formulation comprising a protein in an amount of at least about 50 mg/mL and a diluent, which reconstituted formulation has been prepared from a lyophilized mixture of a protein and a lyoprotectant, wherein the protein concentration in the reconstituted formulation is about 2-40 times greater than the protein concentration in the mixture before lyophilization.

[0011] In another embodiment, the invention provides a stable reconstituted formulation comprising an antibody in an amount of at least about 50 mg/mL and a diluent, which reconstituted formulation has been prepared from a lyophilized mixture of an antibody and a lyoprotectant, wherein the antibody concentration in the reconstituted formulation is about 2-40 times greater than the antibody concentration in the mixture before lyophilization.

[0012] The ratio of lyoprotectant:protein in the lyophilized formulation of the preceding paragraphs depends, for example, on both the protein and lyoprotectant of choice, as well as the desired protein concentration and isotonicity of the reconstituted formulation. In the case of a full length antibody (as the protein) and trehalose or sucrose (as the

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lyoprotectant) for generating a high protein concentration isotonic reconstituted formulation, the ratio may, for example, be about 100-1500 mole trehalose or sucrose: 1 mole antibody.

[0013] Generally, the pre-lyophilized formulation of the protein and lyoprotectant will further include a buffer which provides the formulation at a suitable pH, depending on the protein in the formulation. For this purpose, it has been found to be desirable to use a histidine buffer in that, as demonstrated below, this appears to have lyoprotective properties.

[0014] The formulation may further include a surfactant (e.g. a polysorbate) in that it has been observed herein that this can reduce aggregation of the reconstituted protein and/or reduce the formation of particulates in the reconstituted formulation. The surfactant can be added to the pre-lyophilized formulation, the lyophilized formulation and/or the reconstituted formulation (but preferably the pre-lyophilized formulation) as desired.

[0015] The invention further provides a method for preparing a stable isotonic reconstituted formulation comprising reconstituting a lyophilized mixture of a protein and a lyoprotectant in a diluent such that the protein concentration in the reconstituted formulation is at least 50 mg/mL, wherein the protein concentration in the reconstituted formulation is about 2-40 times greater than the protein concentration in the mixture before lyophilization.

[0016] In yet a further embodiment, the invention provides a method for preparing a formulation comprising the steps of: (a) lyophilizing a mixture of a protein and a lyoprotectant; and (b) reconstituting the lyophilized mixture of step (a) in a diluent such that the reconstituted formulation is isotonic and stable and has a protein concentration of at least about 50 mg/mL. For example, the protein concentration in the reconstituted formulation may be from about 80 mg/mL to about 300 mg/mL. Generally, the protein concentration in the reconstituted formulation is about 2-40 times greater than the protein concentration in the mixture before lyophilization.

[0017] An article of manufacture is also provided herein which comprises: (a) a container which holds a lyophilized mixture of a protein and a lyoprotectant; and (b) instructions for reconstituting the lyophilized mixture with a diluent to a protein concentration in the reconstituted formulation of at least about 50 mg/mL. The article of manufacture may further comprise a second container which holds a diluent (e.g. bacteriostatic water for injection (BWFI) comprising an aromatic alcohol).

[0018] The invention further provides a method for treating a mammal comprising administering a therapeutically effective amount of a reconstituted formulation disclosed herein to a mammal, wherein the mammal has a disorder requiring treatment with the protein in the formulation. For example, the formulation may be administered subcutaneously.

[0019] One useful anti-HER2 antibody pre-lyophilized formulation as discovered in the experiments detailed below was found to comprise anti-HER2 in amount from about 5-40 mg/mL (e.g. 20-30 mg/mL) and sucrose or trehalose in an amount from about 10-100 mM (e.g. 40-80 mM), a buffer (e.g. histidine, pH 6 or succinate, pH 5) and a surfactant (e.g. a polysorbate). The lyophilized formulation was found to be stable at 40° C. for at least 3 months and stable at 30° C. for at least 6 months. This anti-HER2 formulation can be reconstituted with a diluent to generate a formulation suitable for intravenous administration comprising anti-HER2 in an amount from about 10-30 mg/mL which is stable at 2-8° C. for at least about 30 days. Where higher concentrations of the anti-HER2 antibody are desired (for example where subcutaneous delivery of the antibody is the intended mode of administration to the patient), the lyophilized formulation may be reconstituted to yield a stable reconstituted formulation having a protein concentration of 50 mg/mL or more.

[0020] One desirable anti-IgE antibody pre-lyophilized formulation discovered herein has anti-IgE in amount from about 5-40 mg/mL (e.g. 20-30 mg/mL) and sucrose or trehalose in an amount from about 60-300 mM (e.g. 80-170 mM), a buffer (preferably histidine, pH 6) and a surfactant (such as a polysorbate). The lyophilized anti-IgE formulation is stable at 30° C. for at least 1 year. This formulation can be reconstituted to yield a formulation comprising anti-IgE in an amount from about 15-45 mg/mL (e.g. 15-25 mg/mL) suitable for intravenous administration which is stable at 2-8° C. for at least 1 year. Alternatively, where higher concentrations of anti-IgE in the formulation are desired, the lyophilized formulation can be reconstituted in order to generate a stable formulation having an anti-IgE concentration of $\geq$50 mg/mL.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] **FIG. 1** shows the effect of reconstitution volume on the stability of lyophilized rhuMAb HER2. The lyophilized formulation was prepared from a pre-lyophilization formulation comprising 25 mg/mL protein, 60 mM trehalose, 5 mM sodium succinate, pH 5.0, and 0.01% Tween 20™. The lyophilized cake was incubated at 40° C. and then reconstituted with 4.0 (○) or 20.0 mL (●) of BWFI. The fraction of intact protein in the reconstituted formulation was measured by native size exclusion chromatography and defined as the peak area of the native protein relative to the total peak area including aggregates.

[0022] **FIG. 2** illustrates the effect of trehalose concentration on the stability of lyophilized rhuMAb HER2. The protein was lyophilized at 25 mg/mL in 5 mM sodium succinate, pH 5.0 (circles) or 5 mM histidine, pH 6.0 (squares) and trehalose concentrations ranging from 60 mM (360 molar ratio) to 200 mM (1200 molar ratio). The lyophilized protein was incubated at 40° C. for either 30 days (closed symbols) or 91 days (open symbols). The amount of intact protein was measured after reconstitution of the lyophilized protein with 20 mL BWFI.

[0023] **FIG. 3** demonstrates the effect of trehalose concentration on the long term stability of lyophilized rhuMAb HER2 stored at 40° C. The protein was lyophilized at either 25 mg/mL in 5 mM sodium succinate, pH 5.0, 0.01% Tween 20™, and 60 mM trehalose (■) or 5 mM histidine, pH 6.0, 0.01 % Tween 20™, and 60 mM trehalose (□) or 21 mg/mL in 10 mM sodium succinate, pH 5.0, 0.2% Tween 20™ and 250 mM trehalose (●). The lyophilized protein was incubated at 40° C. and then reconstituted with 20 mL of BWFI. The amount of intact protein was measured after reconstitution.

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[0024] FIG. 4 shows the stability of rhuMAb HER2 lyophilized in 38.4 mM mannitol (7 mg/mL), 20.4 mM sucrose (7 mg/mL), 5 mM histidine, pH 6.0, 0.01% Tween 20™. The lyophilized protein was incubated at 40° C. and then reconstituted with either 4.0 mL (○) or 20 mL (●) of BWFI. The amount of intact protein was measured after reconstitution.

[0025] FIG. 5 demonstrates stability of reconstituted rhuMAb HER2 lyophilized in 5 mM sodium succinate, pH5.0, 60 mM trehalose, 0.01% Tween 20™. Samples were reconstituted with either 4.0 mL (squares) or 20.0 mL (circles) of BWFI (20 mL: 0.9% benzyl alcohol; 4 mL: 1.1% benzyl alcohol) and then stored at 5° C. (solid symbols) or 25° C. (open symbols). The % native protein was defined as the peak area of the native (not degraded) protein relative to the total peak area as measured by cation exchange chromatography.

[0026] FIG. 6 shows stability of reconstituted rhuMAb HER2 lyophilized in 5 mM histidine, pH 6.0, 60 mM trehalose, 0.01% Tween 20. Samples were reconstituted with either 4.0 mL (squares) or 20.0 mL (circles) of BWFI (20 mL: 0.9% benzyl alcohol; 4 mL: 1.1% benzyl alcohol) and then stored at 5° C. (solid symbols) or 25° C. (open symbols). The % native protein was defined as the peak area of the native (not degraded) protein relative to the total peak area as measured by cation exchange chromatography.

[0027] FIG. 7 reveals stability of reconstituted rhuMAb HER2 lyophilized in 5 mM histidine, pH 6.0, 38.4 mM mannitol, 20.4 mM sucrose, 0.01% Tween 20. Samples were reconstituted with either 4.0 mL (squares) or 20.0 mL (circles) of BWFI (20 mL: 0.9% benzyl alcohol; 4 mL: 1.1% benzyl alcohol) and then stored at 5° C. (solid symbols) or 25° C. (open symbols). The % native protein was defined as the peak area of the native (not degraded) protein relative to the total peak area as measured by cation exchange chromatography.

[0028] FIG. 8 shows stability of reconstituted rhuMAb HER2 lyophilized in 10 mM sodium succinate, pH 5.0, 250 mM trehalose, 0.2% Tween 20. Samples were reconstituted with 20.0 mL of BWFI (0.9% benzyl alcohol) and then stored at 5° C. (●) or 25 ° C. (○). The % native protein was defined as the peak area of the native (not degraded) protein relative to the total peak area as measured by cation exchange chromatography.

[0029] FIG. 9 shows aggregation of rhuMAb E25 formulated into buffers ranging from pH 5 to pH 7 at 10 mM buffer concentration and 5 mg/mL antibody concentration. Samples were lyophilized and assayed at time zero and after 4 weeks, 8 weeks, and 52 weeks of storage at 2-8° C. The buffers were: potassium phosphate pH 7.0 (○); sodium phosphate pH 7.0 (□); histidine pH 7.0 (◇); sodium succinate pH 6.5 (●); sodium succinate pH 6.0(□); sodium succinate pH 5.5 (◆); and sodium succinate pH5.0 (▲).

[0030] FIG. 10 depicts aggregation of rhuMAb E25 lyophilized in 5 mM histidine buffer at both pH 6 and pH 7 and assayed following storage as follows. The buffer was at: pH 6.0 stored at 2-8° C. (○); pH 6 stored at 25° C. (□); pH 6 stored at 40° C. (◇); pH 7 stored at 2-8° C. (●); pH 7 stored at 25° C. (■); and pH 7 stored at 40° C. (◇).

[0031] FIG. 11 illustrates aggregation of 5 mg/mL rhuMAb E25 formulated into 10 mM sodium succinate at pH 5.0 with lyoprotectant added at a concentration of 275 mM (isotonic). The lyoprotectants were: control, no lyoprotectant (○); mannitol (□); lactose (0); maltose (●); trehalose (■); and sucrose (◆). Samples were lyophilized and assayed at time zero and after 4 weeks, 8 weeks, and 52 weeks of storage at 2-8° C.

[0032] FIG. 12 shows aggregation of 5 mg/mL rhuMAb E25 formulated into 10 mM sodium succinate at pH 5.0 with lyoprotectant added at a concentration of 275 mM (isotonic). The lyoprotectants were: control, no lyoprotectant (○); mannitol (□); lactose (◇); maltose (●); trehalose (■); and sucrose (◆). Samples were lyophilized and assayed at time zero and after 4 weeks, 8 weeks, and 52 weeks of storage at 40° C.

[0033] FIG. 13 depicts hydrophobic interaction chromatography of 20 mg/mL rhuMAb E25 lyophilized in histidine buffer at pH 6 with an isotonic concentration (i.e. 275 mM) of lactose stored for 24 weeks at 2-8, 25 or 40° C. and reconstituted to 20 mg/mL.

[0034] FIG. 14 shows hydrophobic interaction chromatography of 20 mg/mL rhuMAb E25 lyophilized in histidine buffer at pH 6 stored for 24 weeks at 2-8, 25 or 40° C. and reconstituted to 20 mg/mL.

[0035] FIG. 15 illustrates hydrophobic interaction chromatography of 20 mg/mL rhuMAb E25 lyophilized in histidine buffer at pH 6 with an isotonic concentration (i.e. 275 mM) of sucrose and stored for 24 weeks at 2-8, 25 or 40° C. and reconstituted to 20 mg/mL.

[0036] FIG. 16 illustrates the effect of sugar concentration on rhuMAb E25 formulated at 20 mg/mL in 5 mM histidine at pH 6.0. Sucrose (●) and trehalose (□) were added to the formulation at molar ratios ranging from 0 to 2010 (isotonic) (see Table 1 below). Samples were lyophilized and assayed after 12 weeks of storage at 50° C.

TABLE 1

Moles of Sugar: E25 antibody	Sugar conc. (mM)
0	0
260	34.4
380	51.6
510	68.8
760	103.1
1020	137.5
1530	206.3
2010	275

[0037] FIG. 17 reveals aggregation of rhuMAb E25 formulated at 25 mg/mL into 5 mM histidine at pH 6 with 85 mM sucrose (○); 85 mM trehalose (□); 161 mM sucrose (◆) or 161 mM trehalose (▲). Samples were lyophilized and stored at 2-8° C. followed by reconstitution with 0.9% benzyl alcohol to 100 mg/mL antibody in 20 mM histidine at pH 6 with isotonic (340 mM) and hypertonic (644 mM) sugar concentration.

[0038] FIG. 18 shows aggregation of rhuMAb E25 formulated at 25 mg/mL into 5 mM histidine at pH 6 with 85 mM sucrose (○); 85 mM trehalose (□); 161 mM sucrose (◆) or 161 mM trehalose (▲). Samples were lyophilized and stored at 30° C. followed by reconstitution with 0.9% benzyl alcohol to 100 mg/mL antibody in 20 mM histidine at pH 6 with isotonic (340 mM) and hypertonic (644 mM) sugar concentration.

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[0039] **FIG. 19** illustrates aggregation of rhuMAb E25 formulated at 25 mg/mL into 5 mM histidine at pH 6 with 85 mM sucrose (○); 85 mM trehalose (□); 161 mM sucrose (◆) or 161 mM trehalose (▲). Samples were lyophilized and stored at 50° C. followed by reconstitution with 0.9% benzyl alcohol to 100 mg/mL antibody in 20 mM histidine at pH 6 with isotonic (340 mM) and hypertonic (644 mM) sugar concentration.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0040] I. Definitions

[0041] By "protein" is meant a sequence of amino acids for which the chain length is sufficient to produce the higher levels of tertiary and/or quaternary structure. This is to distinguish from "peptides" or other small molecular weight drugs that do not have such structure. Typically, the protein herein will have a molecular weight of at least about 15-20 kD, preferably at least about 20 kD.

[0042] Examples of proteins encompassed within the definition herein include mammalian proteins, such as, e.g., growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; α-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anticlotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or tissue-type plasminogen activator (t-PA); bombazine; thrombin; tumor necrosis factor-α and -β; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-α); serum albumin such as human serum albumin; mullerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; DNase; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; an integrin; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-α and TGF-β, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I); insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19 and CD20; erythropoietin (EPO); thrombopoietin (TPO); osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-α, -β, and -γ; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor (DAF); a viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; immunoadhesins; antibodies; and biologically active fragments or variants of any of the above-listed polypeptides.

[0043] The protein which is formulated is preferably essentially pure and desirably essentially homogeneous (i.e. free from contaminating proteins etc). "Essentially pure" protein means a composition comprising at least about 90% by weight of the protein, based on total weight of the composition, preferably at least about 95% by weight. "Essentially homogeneous" protein means a composition comprising at least about 99% by weight of protein, based on total weight of the composition.

[0044] In certain embodiments, the protein is an antibody. The antibody may bind to any of the above-mentioned molecules, for example. Exemplary molecular targets for antibodies encompassed by the present invention include CD proteins such as CD3, CD4, CD8, CD19, CD20 and CD34; members of the HER receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mo1, p150,95, VLA-4, ICAM-1, VCAM and αv/β3 integrin including either α or β subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; protein C etc.

[0045] The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length antibodies which have an immunoglobulin Fe region), antibody compositions with polyepitopic specificity, bispecific antibodies, diabodies, and single-chain molecules, as well as antibody fragments (e.g., Fab, $F(ab')_2$, and Fv).

[0046] The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature*, 256: 495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., *Nature*, 352:624-628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581-597 (1991), for example.

[0047] The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or

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belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984)).

[0048] "Humanized" forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementarity determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fe), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature*, 321:522-525 (1986); Reichmann et al., *Nature*, 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.*, 2:593-596 (1992). The humanized antibody includes a Primatized™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

[0049] A "stable" formulation is one in which the protein therein essentially retains its physical and chemical stability and integrity upon storage. Various analytical techniques for measuring protein stability are available in the art and are reviewed in *Peptide and Protein Drug Delivery*, 247-301, Vincent Lee Ed., Marcel Dekker, Inc., New York, New York, Pubs. (1991) and Jones, A. *Adv. Drug Delivery Rev.* 10: 29-90 (1993). Stability can be measured at a selected temperature for a selected time period. For rapid screening, the formulation may be kept at 40° C. for 2 weeks to 1 month, at which time stability is measured. Where the formulation is to be stored at 2-8° C., generally the formulation should be stable at 30° C. or 40° C. for at least 1 month and/or stable at 2-8° C. for at least 2 years at 30° C. formulation is to be stored at 30° C., generally the formulation should be stable for at least 2 years at 30° C. and/or stable at 40° C. for at least 6 months. For example, the extent of aggregation following lyophilization and storage can be used as an indicator of protein stability (see Examples herein). For example, a "stable" formulation may be one wherein less than about 10% and preferably less than about 5% of the protein is present as an aggregate in the formulation. In other embodiments, any increase in aggregate formation following lyophilization and storage of the lyophilized formulation can be determined. For example, a "stable" lyophilized formulation may be one wherein the increase in aggregate in the lyophilized formulation is less than about 5% and preferably less than about 3%, when the lyophilized formulation is stored at 2-8° C. for at least one year. In other embodiments, stability of the protein formulation may be measured using a biological activity assay (see, e.g., Example 2 below).

[0050] A "reconstituted" formulation is one which has been prepared by dissolving a lyophilized protein formulation in a diluent such that the protein is dispersed in the reconstituted formulation. The reconstituted formulation in suitable for administration (e.g. parenteral administration) to a patient to be treated with the protein of interest and, in certain embodiments of the invention, may be one which is suitable for subcutaneous administration.

[0051] By "isotonic" is meant that the formulation of interest has essentially the same osmotic pressure as human blood. Isotonic formulations will generally have an osmotic pressure from about 250 to 350 mOsm. Isotonicity can be measured using a vapor pressure or ice-freezing type osmometer, for example.

[0052] A "lyoprotectant" is a molecule which, when combined with a protein of interest, significantly prevents or reduces chemical and/or physical instability of the protein upon lyophilization and subsequent storage. Exemplary lyoprotectants include sugars such as sucrose or trehalose; an amino acid such as monosodium glutamate or histidine; a methylamine such as betaine; a lyotropic salt such as magnesium sulfate; a polyol such as trihydric or higher sugar alcohols, e.g. glycerin, erythritol, glycerol, arabitol, xylitol, sorbitol, and mannitol; propylene glycol; polyethylene glycol; Pluronics; and combinations thereof. The preferred lyoprotectant is a non-reducing sugar, such as trehalose or sucrose.

[0053] The lyoprotectant is added to the pre-lyophilized formulation in a "lyoprotecting amount" which means that, following lyophilization of the protein in the presence of the lyoprotecting amount of the lyoprotectant, the protein essentially retains its physical and chemical stability and integrity upon lyophilization and storage.

[0054] The "diluent" of interest herein is one which is pharmaceutically acceptable (safe and non-toxic for administration to a human) and is useful for the preparation of a reconstituted formulation. Exemplary diluents include sterile water, bacteriostatic water for injection (BWFI), a pH buffered solution (e.g. phosphate-buffered saline), sterile saline solution, Ringer's solution or dextrose solution.

[0055] A "preservative" is a compound which can be added to the diluent to essentially reduce bacterial action in the reconstituted formulation, thus facilitating the production of a multi-use reconstituted formulation, for example. Examples of potential preservatives include octadecyldimethylbenzyl ammonium chloride, hexamethonium chloride, benzalkonium chloride (a mixture of alkylbenzyldimethylammonium chlorides in which the alkyl groups are long-chain compounds), and benzethonium chloride. Other types of preservatives include aromatic alcohols such as phenol, butyl and benzyl alcohol, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol, 3-pentanol, and m-cresol. The most preferred preservative herein is benzyl alcohol.

[0056] A "bulking agent" is a compound which adds mass to the lyophilized mixture and contributes to the physical structure of the lyophilized cake (e.g. facilitates the produc-

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tion of an essentially uniform lyophilized cake which maintains an open pore structure). Exemplary bulking agents include mannitol, glycine, polyethylene glycol and xorbitol.

[0057] "Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

[0058] "Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

[0059] A "disorder" is any condition that would benefit from treatment with the protein. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include carcinomas and allergies.

[0060] II. Modes for Carrying out the Invention

[0061] A. Protein Preparation

[0062] The protein to be formulated is prepared using techniques which are well established in the art including synthetic techniques (such as recombinant techniques and peptide synthesis or a combination of these techniques) or may be isolated from an endogenous source of the protein. In certain embodiments of the invention, the protein of choice is an antibody. Techniques for the production of antibodies follow.

[0063] (i) Polyclonal Antibodies.

[0064] Polyclonal antibodies are generally raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

[0065] Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining 1 mg or 1 μg of the peptide or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant. One month later the animals are boosted with ⅕ to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

[0066] (ii) Monoclonal Antibodies.

[0067] Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

[0068] For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

[0069] In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice,* pp.59-103 (Academic Press, 1986)).

[0070] The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

[0071] Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.,* 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications,* pp.51-63 (Marcel Dekker, Inc., New York, 1987)).

[0072] Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

[0073] The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson et al., *Anal. Biochem.,* 107:220 (1980).

[0074] After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, Monoclonal Antibodies: *Principles and Practice,* pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose

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include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

[0075] The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

[0076] DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al., *Curr. Opinion in Immunol.*, 5:256-262 (1993) and Plückthun, *Immunol. Revs.*, 130:151-188 (1992).

[0077] In a further embodiment, antibodies can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature*, 348:552-554 (1990). Clackson et al., *Nature*, 352:624-628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al, *Bio/Technology*, 10:779-783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.*, 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

[0078] The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, et al., *Proc. Natl Acad. Sci. USA*, 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

[0079] Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

[0080] Chimeric or hybrid antibodies also may be prepared in vitro using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide-exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate.

[0081] (iii) Humanized and Human Antibodies.

[0082] Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*, 332:323-327 (1988); Verhoeyen et al., *Science*, 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No.4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

[0083] The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims et al., *J. Immunol.*, 151:2296 (1993); Chothia et al., *J. Mol. Biol.*, 196:901 (1987)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta et al., *J. Immnol.*, 151:2623 (1993)).

[0084] It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

[0085] Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in

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the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA,* 90:2551 (1993); Jakobovits et al., *Nature,* 362:255-258 (1993); Bruggermann et al., *Year in Immuno.,* 7:33 (1993). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., *J. Mol. Biol.,* 227:381 (1991); Marks et al., *J. Mol. Biol.,* 222:581-597 (1991)).

[0086] (iv) Bispecific Antibodies

[0087] Bispecific antibodies (BsAbs) are antibodies that have binding specificities for at least two different epitopes. Such antibodies can be derived from full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

[0088] Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature,* 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829 and in Traunecker et al., *EMBO J.,* 10:3655-3659 (1991).

[0089] According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

[0090] In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690 published Mar. 3, 1994. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology,* 121:210 (1986).

[0091] Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

[0092] Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. The following techniques can also be used for the production of bivalent antibody fragments which are not necessarily bispecific. For example, Fab' fragments recovered from *E. coli* can be chemically coupled in vitro to form bivalent antibodies. See, Shalaby et al., *J. Exp. Med.,* 175:217-225 (1992).

[0093] Various techniques for making and isolating bivalent antibody fragments directly from recombinant cell culture have also been described. For example, bivalent heterodimers have been produced using leucine zippers. Kostelny et al., *J. Immunol.,* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific/bivalent antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific/bivalent antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.,* 152:5368 (1994).

[0094] B. Preparation of the Lyophilized Formulation

[0095] After preparation of the protein of interest as described above, a "pre-lyophilized formulation" is produced. The amount of protein present in the pre-lyophilized formulation is determined taking into account the desired dose volumes, mode(s) of administration etc. Where the protein of choice is an intact antibody (such as an anti-IgE or anti-HER2 antibody), from about 2 mg/mL to about 50 mg/mL, preferably from about 5 mg/mL to about 40 mg/mL and most preferably from about 20-30 mg/mL is an exemplary starting protein concentration. The protein is generally present in solution. For example, the protein may be present in a pH-buffered solution at a pH from about 4-8, and preferably from about 5-7. Exemplary buffers include his-

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tidine, phosphate, Tris, citrate, succinate and other organic acids. The buffer concentration can be from about 1 mM to about 20 mM, or from about 3 mM to about 15 mM, depending, for example, on the buffer and the desired isotonicity of the formulation (e.g. of the reconstituted formulation). The preferred buffer is histidine in that, as demonstrated below, this can have lyoprotective properties. Succinate was shown to be another useful buffer.

[0096] The lyoprotectant is added to the pre-lyophilized formulation. In preferred embodiments, the lyoprotectant is a non-reducing sugar such as sucrose or trehalose. The amount of lyoprotectant in the pre-lyophilized formulation is generally such that, upon reconstitution, the resulting formulation will be isotonic. However, hypertonic reconstituted formulations may also be suitable. In addition, the amount of lyoprotectant must not be too low such that an unacceptable amount of degradation/aggregation of the protein occurs upon lyophilization. Where the lyoprotectant is a sugar (such as sucrose or trehalose) and the protein is an antibody, exemplary lyoprotectant concentrations in the pre-lyophilized formulation are from about 10 mM to about 400 mM, and preferably from about 30 mM to about 300 mM, and most preferably from about 50 mM to about 100 mM.

[0097] The ratio of protein to lyoprotectant is selected for each protein and lyoprotectant combination. In the case of an antibody as the protein of choice and a sugar (e.g., sucrose or trehalose) as the lyoprotectant for generating an isotonic reconstituted formulation with a high protein concentration, the molar ratio of lyoprotectant to antibody may be from about 100 to about 1500 moles lyoprotectant to 1 mole antibody, and preferably from about 200 to about 1000 moles of lyoprotectant to 1 mole antibody, for example from about 200 to about 600 moles of lyoprotectant to 1 mole antibody.

[0098] In preferred embodiments of the invention, it has been found to be desirable to add a surfactant to the pre-lyophilized formulation. Alternatively, or in addition, the surfactant may be added to the lyophilized formulation and/or the reconstituted formulation. Exemplary surfactants include nonionic surfactants such as polysorbates (e.g. polysorbates 20 or 80); poloxamers (e.g. poloxamer 188); Triton; sodium dodecyl sulfate (SDS); sodium laurel sulfate; sodium octyl glycoside; lauryl-, myristyl-, linoleyl-, or stearyl-sulfobetaine; lauryl-, myristyl-, linoleyl- or stearyl-sarcosine; linoleyl-, myristyl-, or cetyl-betaine; lauroamidopropyl-, cocamidopropyl-, linoleamidopropyl-, myristamidopropyl-, palmidopropyl-, or isostearamidopropyl-betaine (e.g. lauroamidopropyl); myristamidopropyl-, palmidopropyl-, or isostearamidopropyl-dimethylamine; sodium methyl cocoyl-, or disodium methyl oleyl-taurate; and the MONAQUAT™ series (Mona Industries, Inc., Paterson, N.J.), polyethyl glycol, polypropyl glycol, and copolymers of ethylene and propylene glycol (e.g. Pluronics, PF68 etc). The amount of surfactant added is such that it reduces aggregation of the reconstituted protein and minimizes the formation of particulates after reconstitution. For example, the surfactant may be present in the pre-lyophilized formulation in an amount from about 0.001-0.5%, and preferably from about 0.005-0.05%.

[0099] In certain embodiments of the invention, a mixture of the lyoprotectant (such as sucrose or trehalose) and a bulking agent (e.g. mannitol or glycine) is used in the preparation of the pre-lyophilization formulation. The bulking agent may allow for the production of a uniform lyophilized cake without excessive pockets therein etc.

[0100] Other pharmaceutically acceptable carriers, excipients or stabilizers such as those described in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980) may be included in the pre-lyophilized formulation (and/or the lyophilized formulation and/or the reconstituted formulation) provided that they do not adversely affect the desired characteristics of the formulation. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed and include; additional buffering agents; preservatives; co-solvents; antioxidants including ascorbic acid and methionine; chelating agents such as EDTA; metal complexes (e.g. Zn-protein complexes); biodegradable polymers such as polyesters; and/or salt-forming counterions such as sodium.

[0101] The formulation herein may also contain more than one protein as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect the other protein. For example, it may be desirable to provide two or more antibodies which bind to the HER2 receptor or IgE in a single formulation. Furthermore, anti-HER2 and anti-VEGF antibodies may be combined in the one formulation. Such proteins are suitably present in combination in amounts that are effective for the purpose intended.

[0102] The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to, or following, lyophilization and reconstitution. Alternatively, sterility of the entire mixture may be accomplished by autoclaving the ingredients, except for protein, at about 120° C. for about 30 minutes, for example.

[0103] After the protein, lyoprotectant and other optional components are mixed together, the formulation is lyophilized. Many different freeze-dryers are available for this purpose such as Hull50™ (Hull, USA) or GT20™ (Leybold-Heraeus, Germany) freeze-dryers. Freeze-drying is accomplished by freezing the formulation and subsequently subliming ice from the frozen content at a temperature suitable for primary drying. Under this condition, the product temperature is below the eutectic point or the collapse temperature of the formulation. Typically, the shelf temperature for the primary drying will range from about −30 to 25° C. (provided the product remains frozen during primary drying) at a suitable pressure, ranging typically from about 50 to 250 mTorr. The formulation, size and type of the container holding the sample (e.g., glass vial) and the volume of liquid will mainly dictate the time required for drying, which can range from a few hours to several days (e.g. 40-60 hrs). A secondary drying stage may be carried out at about 0-40° C., depending primarily on the type and size of container and the type of protein employed. However, it was found herein that a secondary drying step may not be necessary. For example, the shelf temperature throughout the entire water removal phase of lyophilization may be from about 15-30° C. (e.g., about 20° C.). The time and pressure required for secondary drying will be that which produces a suitable lyophilized cake, dependent, e.g., on the temperature and other parameters. The secondary drying time is dictated by the desired residual moisture level in the

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product and typically takes at least about 5 hours (e.g. 10-15 hours). The pressure may be the same as that employed during the primary drying step. Freeze-drying conditions can be varied depending on the formulation and vial size.

[0104] In some instances, it may be desirable to lyophilize the protein formulation in the container in which reconstitution of the protein is to be carried out in order to avoid a transfer step. The container in this instance may, for example, be a 3, 5, 10, 20, 50 or 100 cc vial.

[0105] As a general proposition, lyophilization will result in a lyophilized formulation in which the moisture content thereof is less than about 5%, and preferably less than about 3%.

[0106] C. Reconstitution of the Lyophilized Formulation

[0107] At the desired stage, typically when it is time to administer the protein to the patient, the lyophilized formulation may be reconstituted with a diluent such that the protein concentration in the reconstituted formulation is at least 50 mg/mL, for example from about 50 mg/mL to about 400 mg/mL, more preferably from about 80 mg/mL to about 300 mg/mL, and most preferably from about 90 mg/mL to about 150 mg/mL. Such high protein concentrations in the reconstituted formulation are considered to be particularly useful where subcutaneous delivery of the reconstituted formulation is intended. However, for other routes of administration, such as intravenous administration, lower concentrations of the protein in the reconstituted formulation may be desired (for example from about 5-50 mg/mL, or from about 10-40 mg/mL protein in the reconstituted formulation). In certain embodiments, the protein concentration in the reconstituted formulation is significantly higher than that in the pre-lyophilized formulation. For example, the protein concentration in the reconstituted formulation may be about 2-40 times, preferably 3-10 times and most preferably 3-6 times (e.g. at least three fold or at least four fold) that of the pre-lyophilized formulation.

[0108] Reconstitution generally takes place at a temperature of about 25° C. to ensure complete hydration, although other temperatures maybe employed as desired. The time required for reconstitution will depend, e.g. on the type of diluent, amount of excipient(s) and protein. Exemplary diluents include sterile water, bacteriostatic water for injection (BWFI), a pH buffered solution (e.g. phosphate-buffered saline), sterile saline solution, Ringer's solution or dextrose solution. The diluent optionally contains a preservative. Exemplary preservatives have been described above, with aromatic alcohols such as benzyl or phenol alcohol being the preferred preservatives. The amount of preservative employed is determined by assessing different preservative concentrations for compatibility with the protein and preservative efficacy testing. For example, if the preservative is an aromatic alcohol (such as benzyl alcohol), it can be present in an amount from about 0.1-2.0% and preferably from about 0.5-1.5%, but most preferably about 1.0-1.2%.

[0109] Preferably, the reconstituted formulation has less than 6000 particles per vial which are ≥ 10 μm in size.

[0110] D. Administration of the Reconstituted Formulation

[0111] The reconstituted formulation is administered to a mammal in need of treatment with the protein, preferably a human, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes.

[0112] In preferred embodiments, the reconstituted formulation is administered to the mammal by subcutaneous (i.e. beneath the skin) administration. For such purposes, the formulation may be injected using a syringe. However, other devices for administration of the formulation are available such as injection devices (e.g. the Inject-ease™ and Genject™ devices); injector pens (such as the GenPen™); needleless devices (e.g. MediJector™ and BioJector™); and subcutaneous patch delivery systems.

[0113] The appropriate dosage ("therapeutically effective amount") of the protein will depend, for example, on the condition to be treated, the severity and course of the condition, whether the protein is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the protein, the type of protein used, and the discretion of the attending physician. The protein is suitably administered to the patient at one time or over a series of treatments and may be administered to the patient at any time from diagnosis onwards. The protein may be administered as the sole treatment or in conjunction with other drugs or therapies useful in treating the condition in question.

[0114] Where the protein of choice is an antibody, from about 0.1-20 mg/kg is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques.

[0115] In the case of an anti-HER2 antibody, a therapeutically effective amount of the antibody may be administered to treat or prevent cancer characterized by overexpression of the HER2 receptor. It is contemplated that a reconstituted formulation of the anti-HER2 antibody may be used to treat breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon and/or bladder cancer. For example, the anti-HER2 antibody may be used to treat ductal carcinoma in situ (DCIS). Exemplary dosages of the anti-HER2 antibody are in the range 1-10 mg/kg by one or more separate administrations.

[0116] Uses for an anti-IgE formulation include the treatment or prophylaxis of IgE-mediated allergic diseases, parasitic infections, interstitial cystitis and asthma, for example. Depending on the disease or disorder to be treated, a therapeutically effective amount (e.g. from about 1-15 mg/kg) of the anti-IgE antibody is administered to the patient.

[0117] E. Articles of Manufacture

[0118] In another embodiment of the invention, an article of manufacture is provided which contains the lyophilized formulation of the present invention and provides instructions for its reconstitution and/or use. The article of manufacture comprises a container. Suitable containers include, for example, bottles, vials (e.g. dual chamber vials), syringes (such as dual chamber syringes) and test tubes. The container may be formed from a variety of materials such as glass or plastic. The container holds the lyophilized formu-

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lation and the label on, or associated with, the container may indicate directions for reconstitution and/or use. For example, the label may indicate that the lyophilized formulation is reconstituted to protein concentrations as described above. The label may further indicate that the formulation is useful or intended for subcutaneous administration. The container holding the formulation may be a multi-use vial, which allows for repeat administrations (e.g. from 2-6 administrations) of the reconstituted formulation. The article of manufacture may further comprise a second container comprising a suitable diluent (e.g. BWFI). Upon mixing of the diluent and the lyophilized formulation, the final protein concentration in the reconstituted formulation will generally be at least 50 mg/mL. The article of manufacture may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

[0119] The invention will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the invention. All literature citations are incorporated by reference.

EXAMPLE 1

[0120] Anti-HER2 Formulation

[0121] Overexpression of the HER2 proto-oncogene product ($p185^{HER2}$) has been associated with a variety of aggressive human malignancies. The murine monoclonal antibody known as muMAb4D5 is directed against the extracellular domain (ECD) of $p185^{HER2}$. The muMAb4D5 molecule has been humanized in an attempt to improve its clinical efficacy by reducing immunogenicity and allowing it to support human effector functions (see WO 92/22653). This example describes the development of a lyophilized formulation comprising full length humanized antibody huMAb4D5-8 described in WO 92/22653.

[0122] In the development of a lyophilized formulation, excipients and buffers are initially screened by measuring the stability of the protein after lyophilization and reconstitution. The lyophilized protein in each formulation is also subjected to accelerated stability studies to determine the potential stability of the protein over its shelf-life. These accelerated studies are usually performed at temperatures above the proposed storage conditions and the data are then used to estimate the activation energy for the degradation reactions assuming Arrhenius kinetics (Cleland et al., *Critical Reviews in Therapeutic Drug Carrier Systems* 10(4): 307-377 (1993)). The activation energy is then used to calculate the expected shelf-life of the protein formulation at the proposed storage conditions.

[0123] In early screening studies, the stability of several lyophilized recombinant humanized anti-HER2 antibody (rhuMAb HER2) formulations was investigated after incubation at 5° C. (proposed storage condition) and 40° C. (accelerated stability condition). In the liquid state, rhuMAb HER2 was observed to degrade by deamidation (30Asn of light chain) and isoaspartate formation via a cyclic imide intermediate, succinimide (102Asp of heavy chain). The deamidation was minimized at pH 5.0 resulting in degradation primarily at the succinimide. At pH 6.0, slightly greater deamidation was observed in the liquid protein formulation. The lyophilized formulations were therefore studied with: (a) 5 or 10 mM succinate buffer, pH 5.0 or (b) 5 or 10 mM histidine buffer, pH 6.0. Both buffers contained the surfactant, polysorbate 20 (Tween 20™), which was employed to reduce the potential for aggregation of the reconstituted protein and minimize the formation of particulates after reconstitution. These buffers were used with and without various sugars. The protein was formulated in the buffer at 5.0, 21.0 or 25.0 mg/mL. These formulations were then lyophilized and assessed for protein stability after 2 weeks at 5° C. and 40° C. In the lyophilizer, the vials were frozen at a shelf temperature of −55° C. for approximately 5 hours followed by primary drying at a shelf temperature of 5° C. and 150 mTorr for 30 hours, and drying to 1-2% residual moisture was achieved with secondary drying at a shelf temperature of 20° C. for 10 hours. The major degradation route for this protein upon lyophilization was aggregation, and therefore the protein stability was assessed by native size exclusion chromatography to measure the recovery of intact native protein (% intact protein in Table 2 below).

[0124] The stabilizing effects of various lyoprotectant sugars on lyophilized protein was measured in 10 mM sodium succinate, pH 5.0 (Table 2). At high sugar concentrations (250-275 mM) and low protein concentration (5.0 mg/mL), trehalose and lactose stabilized the protein against aggregation for the lyophilized protein stored for 2 weeks at 40° C. However, lactose, a reducing sugar, was observed to react with the protein over longer term storage at 40° C. The formulations at 5.0 mg/mL protein containing either sorbitol or mannitol yielded aggregated protein after storage at 40° C. for 2 weeks. At the higher protein concentration (21.0 mg/mL), formulations comprising mannitol, or mannitol in combination with sorbitol or glycine, contained aggregated protein after lyophilization and storage at both conditions. In contrast, trehalose and sucrose prevented aggregation at both storage conditions.

[0125] The 250 mM trehalose and 250 mM lactose formulations were assessed for long term stability. After 9 months at 40° C. or 12 months at 5° C., there was no change in the % intact protein for the trehalose formulation. For the lactose formulation, the % intact protein remained constant (same as initial) after 3 months at 40° C. or 6 months at 25° C. The trehalose formulation could be stored at controlled room temperature (15-30° C.) for 2 years without a significant change in % intact protein.

[0126] The 10 mM histidine, pH 6.0 formulation with mannitol contained less aggregated protein after storage at 40° C. for 2 weeks than the 10 mM succinate formulation, pH 5.0 with mannitol. This result may be related to some stabilizing effect contributed by histidine alone. After storage at 40° C. for 2 weeks, there was, however, significant aggregation for histidine alone or histidine/mannitol formulations. The addition of sucrose at an equal mass to mannitol (10 mg/mL of each) in the histidine formulation stabilized the protein against aggregation for both storage conditions. The use of glycine with mannitol did not improve the protein stability, while the sucrose/glycine formulation provided the same stability as the sucrose/mannitol formulation. These results further indicated that sucrose was useful for preventing aggregation of the lyophilized protein during storage.

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TABLE 2

Composition Prior to Lyophilization		% Intact Protein[a]		
[Protein][b] (mg/mL)	Formulation	Liquid (5° C.)	Lyophilized (2 wk, 5° C.)	Lyophilized (2 wk, 40° C.)
	10 mM sodium succinate pH 5.0			
5.0	275 mM trehalose, 0.01% Tween 20 ™	98.9	99.1	98.9
5.0	275 mM lactose, 0.01% Tween 20 ™	96.8	96.5	96.6
5.0	275 mM sorbitol, 0.01% Tween 20 ™	99.4	99.3	95.4
5.0	250 mM mannitol, 0.01% Tween 20 ™	100.0	99.9	98.8
5.0	250 mM trehalose, 0.01% Tween 20 ™	100.0	99.9	100.0
5.0	250 mM lactose, 0.01% Tween 20 ™	100.0	100.0	100.0
21.0	250 mM trehalose, 0.2% Tween 20 ™	99.3	99.1	99.1
21.0	250 mM sucrose, 0.2% Tween 20 ™	99.6	99.6	99.7
21.0	250 mM mannitol, 0.01% Tween 20 ™	100.0	94.6	94.0
21.0	188 mM mannitol/ 63 mM sorbitol, 0.01% Tween 20 ™	99.8	98.6	96.5
21.0	250 mM mannitol/ 25 mM glycine, 0.01% Tween 20 ™	99.5	96.5	96.4
	10 mM histidine pH 6.0			
21.0	No sugar, 0.01% Tween 20 ™	100.0	99.9	98.9
21.0	54.9 mM mannitol, 0.01% Tween 20 ™	100.0	99.9	99.2
21.0	29.2 mM sucrose/ 266.4 mM glycine, 0.01% Tween 20 ™	100.0	100.0	99.6
21.0	54.9 mM mannitol/ 266.4 mM glycine, 0.01% Tween 20 ™	100.0	99.8	98.9
21.0	54.9 mM mannitol/ 29.2 mM sucrose, 0.01% Tween 20 ™	99.8	100.0	99.7

[0127] The delivery of a high protein concentration is often required for subcutaneous administration due to the volume limitations (≦1.5 mL) and dosing requirements (≧100 mg). However, high protein concentrations (≧50 mg/mL) are often difficult to achieve in the manufacturing process since at high concentrations, the protein has a tendency to aggregate during processing and becomes difficult to manipulate (e.g. pump) and sterile filter. Alternatively, the lyophilization process may provide a method to allow concentration of the protein. For example, the protein is filled into vials at a volume (Vf) and then lyophilized. The lyophilized protein is then reconstituted with a smaller volume (Vr) of water or preservative (e.g. BWFI) than the original volume (e.g. Vr=0.25 Vf) resulting in a higher protein concentration in the reconstituted solution. This process also results in the concentration of the buffers and excipients. For subcutaneous administration, the solution is desirably isotonic.

[0128] The amount of trehalose in the lyophilized rhuMAb HER2 was reduced to produce an isotonic solution upon reconstitution to yield 100 mg/mL protein. The stabilizing effect of trehalose was determined as a function of concentration for 5 mM sodium succinate, pH 5.0 and 5 mM histidine, pH 6.0 at 25.0 mg/mL protein (Table 3). At trehalose concentrations from 60 to 200 mM, there was no significant aggregation after incubation of the lyophilized protein for 4 weeks at 40° C. These formulations were reconstituted with 20 mL of bacteriostatic water for injection (BWFI, USP, 0.9% benzyl alcohol). Reconstitution of the 50 mM trehalose formulation (5 mM sodium succinate) with 4 mL of BWFI (100 mg/mL protein) after incubation for 4 weeks at 40° C. yielded a slight increase in aggregate formation. The preserved reconstituted formulations provided the advantage of multiple withdrawals from the same vial without sterility concerns. When sterile needles are used, these formulations would then allow for several doses from a single vial.

TABLE 3

Composition Prior to Lyophilization		% Intact Protein[a]		
[Protein][b] (mg/mL)	Formulation	Liquid (5° C.)	Lyophilized (4 wk, 5° C.)	Lyophilized (4 wk, 40° C.)
	5 mM sodium succinate pH 5.0			
25.0	50 mM trehalose, 0.01% Tween 20 ™[b]	100.00	100.0	99.5
25.0	60 mM trehalose, 0.01% Tween 20 ™	100.0	100.0	99.9
25.0	60 mM trehalose, 0.01% Tween 20 ™	100.0	100.0	99.2
25.0	100 mM trehalose, 0.01% Tween 20 ™	100.0	100.0	99.7
25.0	150 mM trehalose, 0.01% Tween 20 ™	100.0	100.0	99.8
25.0	200 mM trehalose, 0.01% Tween 20 ™	100.0	100.0	100.0
	5 mM histidine pH 6.0			
25.0	38.4 mM mannitol/ 20.4 mM sucrose, 0.01% Tween 20 ™	100.0	100.0	99.3
25.0	38.4 mM mannitol/ 20.4 mM sucrose, 0.01% Tween 20 ™[c]	100.0	100.0	99.4
25.0	60 mM trehalose, 0.01% Tween 20 ™[d]	100.0	100.0	99.8
25.0	60 mM trehalose, 0.01% Tween 20 ™	100.0	100.0	99.4
25.0	100 mM trehalose, 0.01% Tween 20 ™	100.0	100.0	99.6
25.0	150 mM trehalose, 0.01% Tween 20 ™	100.0	100.0	100.0
25.0	200 mM trehalose, 0.01% Tween 20 ™	100.0	100.0	100.0

[a]The fraction of intact protein was measured by native size exclusion HPLC and defined as the peak area of the native protein relative to the total peak area including aggregates (TSK3000 SW XL column, TosoHaas, with a flow rate of 1.0 mL/min; elution with phosphate buffered saline; detection at 214 and 280 nm). The protein formulations were analyzed before lyophilization (liquid, 5° C.) and after lyophilization and storage at 5° C. or 40° C. for 4 weeks. Formulations were reconstituted with bacteriostatic water for injection (BWFI, USP, 0.9% w/w benzyl alcohol; 20 mL, 22 mg/mL protein).
[b] Reconstituted with 4 mL of BWFI (0.9% benzyl alcohol) to yield 100 mg/mL protein.
[c] Reconstituted with 4 mL of BWFI (1.1% benzyl alcohol) to yield 100 mg/mL protein.
[d] Sample incubated for 2 weeks at 5° C. or 40° C. and then reconstituted with 20 mL of BWFI (0.9% benzyl alcohol) to yield 22 mg/mL protein.

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[0129] Currently, rhuMAb HER2 is under investigation as a therapeutic for the treatment of breast cancer. The protein is dosed to patients at 2 mg/kg on a weekly basis. Since the average weight of these patients is 65 kg, the average weekly dose is 130 mg of rhuMAb HER2. For subcutaneous administration, injection volumes of 1.5 mL or less are well tolerated and, therefore, the protein concentration for a weekly subcutaneous administration of rhuMAb HER2 may be approximately 100 mg/mL (130 mg average dose/1.5 mL). As mentioned above, this high protein concentration is difficult to manufacture and maintain in a stable form. To achieve this high protein concentration, rhuMAb HER2 formulated in: (a) 5 mM sodium succinate, pH 5.0 or (b) 5 mM histidine, pH 6.0, was lyophilized at 25 mg/mL protein in 60 mM trehalose, 0.01% Tween 20™. The lyophilization was performed by filling 18 mL of the protein formulation into 50 cc vials. In the lyophilizer, the vials were frozen at a shelf temperature of −55° C. for approximately 5 hours followed by primary drying at a shelf temperature of 5° C. and 150 mTorr for 30 hours, and drying to 1-2% residual moisture was achieved with secondary drying at a shelf temperature of 20° C. for 10 hours. Thermocouples placed in vials containing the placebo (formulation without protein) indicated that the product in the vials was maintained below −10° C. throughout primary drying. Sequential stoppering studies during the lyophilization revealed that the residual moisture after primary drying was usually less than 10%.

[0130] The lyophilized protein was then reconstituted with either 4 or 20 mL of BWFI (0.9 or 1.1% benzyl alcohol) to yield concentrated protein solutions:

[0131] (a) 4 mL: 102 mg/mL rhuMAb HER2, 245 mM trehalose, 21 mM sodium succinate, pH 5.0 or 21 mM histidine, pH 6.0, 0.04% Tween 20™;

[0132] (b) 20 mL: 22 mg/mL rhuMAb HER2, 52 mM trehalose, 4 mM sodium succinate, pH 5.0 or 4 mM histidine, pH 6.0, 0.009% Tween 20™.

[0133] After storage of the lyophilized formulations for 4 weeks at 40° C. and reconstitution to 22 mg/mL protein, the amount of aggregated protein appeared to increase slightly with decreasing trehalose concentration. The stability of the lyophilized protein was not affected by the volume of reconstitution. As shown in **FIG. 1**, the amount of intact protein after incubation of the lyophilized protein at 40° C. was the same for the 60 mM trehalose, 5 mM sodium succinate, pH 5.0, 0.01% Tween 20™ formulation reconstituted with either 4 or 20 mL of BWFI.

[0134] The results shown in Table 3 suggested that there may be a relationship between the trehalose concentration and the protein stability. To further assess this relationship, the formulations containing different concentrations of trehalose formulated in either sodium succinate or histidine were incubated for 91 days at 40° C. The stability was then measured as a function of the trehalose to protein molar ratio for each concentration of trehalose. As shown in **FIG. 2**, the protein stability clearly decreased with decreasing trehalose concentration for both formulations. There was no apparent difference between the two buffers, succinate and histidine, in these formulations suggesting that the primary stabilizer under these conditions is trehalose. In addition, the observed decrease in intact protein for both these formulations would be acceptable even at the low trehalose concentration for a formulation that is stored at 2-8° C. throughout its shelf-life. However, if controlled room temperature (30° C. maximum temperature) stability is required, higher trehalose concentrations (≧600:1 trehalose:protein) may be needed depending on the stability specifications for the product (i.e. the specification for the amount of intact protein remaining after 2 years of storage). Typically, a controlled room temperature storage condition would require stability for 6 months at 40° C. which is equivalent to storage at 30° C. for 2 years

[0135] As shown in **FIG. 3**, the 250 mM trehalose formulation was unchanged after 6 months at 40° C. while both the 60 mM trehalose formulations were less stable. The 60 mM trehalose formulations may then require refrigerated storage if the product specification at the end of its shelf-life is, for example, >98% intact protein by native size exclusion chromatography.

[0136] In the previous screening study, sucrose was also observed to prevent aggregation of rhuMAb HER2 after lyophilization and subsequent storage. To achieve isotonic solutions after reconstitution for subcutaneous administration (approximately four-fold concentration of formulation components and protein), the sucrose concentration must be reduced significantly. The equal mass concentration of sucrose and mannitol (bulking agent) used in the screening studies prevented aggregation of the protein. A lower concentration of sucrose and mannitol (equal mass concentrations) was chosen as a potential subcutaneous formulation of rhuMAb HER2. The protein solution (25 mg/mL protein, 5 mM histidine, pH 6.0, 38.4 mM (7 mg/mL) mannitol, 20.4 mM (7 mg/mL) sucrose, 0.01% Tween 20™) was lyophilized in the same manner as the 60 mM trehalose formulation except that the primary drying cycle was extended to 54 hours. After 4 weeks at 40° C., there was a slight increase in the amount of aggregates after reconstitution with 4.0 or 20.0 mL of BWFI (Table 3). The amount of aggregated protein was the same for reconstitution at 22 or 100 mg/mL protein (**FIG. 4**). Like the 60 mM trehalose formulations, the mannitol/sucrose formulation yielded less intact protein over time at 40° C. The molar ratio of sucrose to protein for this formulation was 120 to 1, indicating that the mannitol/sucrose combination may be more effective than trehalose alone at the same molar ratio of stabilizing sugar (**FIGS. 2 and 4**).

[0137] In the previous examples, the stability of the lyophilized rhuMAb HER2 formulations was determined as a function of temperature. These studies demonstrated that the trehalose and mannitol/sucrose formulations prevented degradation of the protein in the lyophilized state at high temperatures (40° C.). However, these experiments did not address the stability of the protein after reconstitution and storage. Once reconstituted with BWFI, the lyophilized rhuMAb HER2 formulations may be used for several administrations of the drug. In particular, the vial configuration (450 mg rhuMAb HER2) was designed to provide three doses to the average patient (130 mg rhuMAb HER2 per dose). Since the drug is dosed weekly, the vial may be stored at least three weeks after reconstitution. To assure that the rhuMAb HER2 remained stable after reconstitution, stability studies on the reconstituted rhuMAb HER2 formulations were performed at 5° C. and 25° C.

[0138] For subcutaneous administration, the formulations were reconstituted to 100 mg/mL (4 mL BWFI). At this high

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protein concentration, the protein may be more susceptible to aggregation than the intravenous dosage form that was reconstituted to 22 mg/mL protein (20 mL BWFI). The four rhuMAb HER2 formulations from the previous example were assessed for aggregation (loss of intact protein). As shown in Tables 4 through 6, there was no difference in stability for formulations reconstituted at 22 and 100 mg/mL protein. Furthermore, these formulations maintained the protein completely intact for up to 90 days at 5 ° C. and 30 days at 25 ° C., indicating that the reconstituted protein could be stored refrigerated for at least 90 days. Unlike the lyophilized protein stability in the previous example, the trehalose concentration in the formulation did not affect the protein stability (Table 7).

TABLE 4

Stability of the reconstituted formulations for rhuMAb HER2 lyophilized at 25 mg/mL protein in 5 mM sodium succinate, pH 5.0, 60 mM trehalose, 0.01% Tween 20 ™

Time	% Intact Protein			
	22 mg/mL protein		100 mg/mL protein	
(days)	5° C.	25° C.	5° C.	25° C.
0	99.9	99.9	99.7	99.7
14	ND	100.0	ND	100.0
30	100.0	100.0	100.0	100.0
91	99.8	ND	100	ND

The samples were reconstituted with 4.0 or 20.0 mL of BWFI (1.1% or 0.9% benzyl alcohol), and then stored at 5° C. or 25° C. The % intact protein was defined as the fraction of native peak area as measured by native size exclusion chromatography. ND = not determined.

[0139]

TABLE 5

Stability of the reconstituted formulations for rhuMAb HER2 lyophilized at 25 mg/mL protein in 5 mM histidine, pH 6.0, 60 mM trehalose, 0.01% Tween 20 ™

Time	% Intact Protein			
	22 mg/mL protein		100 mg/mL protein	
(days)	5° C.	25° C.	5° C.	25° C.
0	100.0	100.0	100.0	100.0
14	ND	100.0	ND	100.0
31	99.3	99.7	100.0	100.0
61	100.0	ND	ND	ND

The samples were reconstituted with 4.0 or 20.0 mL of BWFI (1.1% or 0.9% benzyl alcohol), and then stored at 5° C. or 25° C. The % intact protein was defined as the fraction of native peak area as measured by native size exclusion chromatography. ND = not determined.

[0140]

TABLE 6

Stability of the reconstituted formulations for rhuMAb HER2 lyophilized at 25 mg/mL protein in 5 mM histidine, pH 6.0, 38.4 mM mannitol, 20.4 mM sucrose, 0.01% Tween 20 ™

Time	% Intact Protein			
	22 mg/mL protein		100 mg/mL protein	
(days)	5° C.	25° C.	5° C.	25° C.
0	99.7	99.7	99.8	99.8
14	ND	100.0	ND	99.8
31	100.0	100.0	100.0	100.0
92	100.0	ND	100.0	ND

The samples were reconstituted with 4.0 or 20.0 mL of BWFI (1.1% or 0.9% benzyl alcohol), and then stored at 5° C. or 25° C. The % intact protein was defined as the fraction of native peak area as measured by native size exclusion chromatography. ND = not determined.

[0141]

TABLE 7

Stability of the reconstituted formulations for rhuMAb HER2 lyophilized at 21 mg/mL protein in 10 mM sodium succinate, pH 5.0, 250 mM trehalose, 0.2% Tween 20 ™

Time	% Intact Protein 21 mg/mL protein	
(days)	5° C.	25° C.
0	99.8	99.8
14	ND	100.0
31	99.9	99.4
92	99.8	ND

The samples were reconstituted with 20.0 mL of BWFI (0.9% benzyl alcohol), and then stored at 5° C. or 25° C. The % intact protein was defined as the fraction of native peak area as measured by native size exclusion chromatography. ND = not determined.

[0142] As mentioned previously, the major degradation route for rhuMAb HER2 in aqueous solutions is deamidation or succinimide formation. The loss of native protein due to deamidation or succinimide formation was assessed for the four reconstituted rhuMAb HER2 formulations.

[0143] Analysis of rhuMAb HER2 deamidation and succinimide formation was performed using cation exchange chromatography. A Bakerbond Wide-Pore Carboxy Sulfon (CSX) column (4.6×250 mm) was operated at a flow rate of 1 mL/min. The mobile phase buffers were (A) 0.02 M sodium phosphate, pH 6.9, and (B) 0.02 M sodium phosphate, pH 6.9, 0.2 M NaCl. The chromatography was then performed at 40° C. as follows:

TABLE 8

Time (min)	% Buffer B
0	10
55	45
57	100
62	100
62.1	10
63	10

Peak elution was monitored at 214 nm and 75 μg of protein was loaded for each analysis.

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[0144] Again, there were no differences in stability of the formulations reconstituted at 22 and 100 mg/mL protein (**FIGS. 5 through 7**). The protein degradation was more rapid at 25° C. than 5° C. for each formulation, and the rate of degradation was comparable for all the formulations stored at 5° C. The formulations containing histidine underwent a slightly greater rate of degradation at 25° C. than the succinate formulations. The amount of trehalose in the formulation did not affect the degradation rate at either temperature (**FIGS. 5 and 8**). These results indicated that these four formulations provide an acceptable rate of degradation under refrigerated storage conditions (5° C.) for the intended period of use (30 days after reconstitution with BWFI).

[0145] Multi-use formulations should pass preservative efficacy testing as described by the US Pharmacopeia (USP) for use in the United States. The rhuMAb HER2 lyophilized formulation consisting of 25 mg/mL protein, 5 mM histidine, pH 6.0, 60 mM trehalose, 0.01% Tween 20™ was reconstituted with 20 mL of benzyl alcohol at concentrations between 0.9 and 1.5% w/w. For concentrations at or above 1.3% w/w, the reconstituted solution became cloudy after overnight incubation at room temperature (~25 ° C.). Reconstitution with the standard BWFI solution (0.9% benzyl alcohol) resulted in a solution that did not consistently pass the preservative challenge tests. However, reconstitution with 1.0 or 1.1% benzyl alcohol was both compatible with the formulation and passed the preservative challenge testing. The manufacturer's specifications for the solution required a range of ±10%, and therefore, the lyophilized formulations are reconstituted with 1.1% benzyl alcohol (1.1±0.1%).

[0146] A single step lyophilization cycle for the rhuMAb HER2 formulation was developed. In the single step lyophilization cycle, rhuMAb HER2 at 25 mg/mL, 60 mM trehalose, 5 mM histidine pH 6 and 0.01% polysorbate 20 was lyophilized at a shelf temperature of 20° C., and a pressure of 150 mTorr. After 47 hours, the residual moisture content of the lyophilized cake was less than 5%. This lyophilization cycle is considered to be useful in that it simplifies the manufacturing process, by eliminating the secondary drying step.

EXAMPLE 2

[0147] Anti-IgE Formulation

[0148] IgE antibodies bind to specific high-affinity receptors on mast cells, leading to mast cell degranulation and release of mediators, such as histamine, which produce symptoms associated with allergy. Hence, anti-IgE antibodies that block binding of IgE to its high-affinity receptor are of potential therapeutic value in the treatment of allergy. These antibodies must also not bind to IgE once it is bound to the receptor because this would trigger histamine release. This example describes the development of a lyophilized formulation comprising full length humanized anti-IgE antibody MaE11 described in Presta et al. *J. Immunology*, 151: 2623-2632 (1993).

[0149] Materials: Highly purified rhuMAb E25 (recombinant humanized anti-IgE antibody MaE11) which did not contain Tween 20™ was used in the formulations described below. Spectra/Por7 dialysis membranes were purchased from Spectrum (Los Angeles, Calif.). All other reagents used in this study were obtained from commercial sources and were of analytical grade. Formulation buffers and chromatography mobile phase were prepared by mixing the appropriate amount of buffer and salt with Milli-Q water in a volumetric flask.

[0150] Formulation: E25 S Sepharose pool was dialyzed into formulation buffers as specified. Dialysis was accomplished by a minimum of 4×2L buffer exchanges over a 48 hour period at 2-8° C. Following dialysis, lyoprotectant was added at a isotonic concentration to some of the formulations as required. Protein concentration following dialysis was determined by UV spectroscopy using a molar absorptivity of 1.60. The dialyzed protein was diluted to the predetermined formulation concentration with an appropriate formulation buffer, sterile filtered using a 0.22 μm Millex-GV filter (Millipore) and dispensed into pre-washed and autoclaved glass vials. The vials were fitted with siliconized teflon lyophilization stoppers and lyophilized using the following conditions: the E25 formulation was frozen to −55° C. at 80° C./hour and the vial content was kept frozen for 4 hours. The shelf temperature was ramped to 25° C. at 10° C./hour for primary drying. Primary drying was carried out at 25° C., 50μ chamber vacuum pressure for 39 hours such that the residual moisture of the lyophilized cake was 1-2%. Following lyophilization, a vial of each formulation was removed for t=0 analysis and the remaining vials were held at various temperatures which include −70° C., 2-8° C., 25° C., 30° C. (controlled room temperature) 40° C. and 50° C.

[0151] Chromatography: Native size exclusion chromatography was carried out on a Bio-Rad Bio-Select™ SEC 250-5 column (300×7.8 mm). The column was equilibrated and ran in PBS at a flow rate of 0.5 mL/min using a Hewlett Packard 1090L HPLC equipped with a diode array detector. Molecular weight standards (Bio-Rad, Inc.) consisting of thyroglobulin (670 kd), gamma-globulin (158 kd), ovalbumin (44 kd), and cyanocobalamin (1.35 kd) were used to calibrate the column. The sample load was 25 μg and protein was detected by monitoring the UV absorption at 214 nm using Turbochrom 3 software (PE Nelson, Inc).

[0152] Hydrophobic Interaction Chromatography: $F(ab')_2$ fragments of the E25 antibody were chromatographed using a TosoHaas Butyl-NPR column (3.5×4.6 mm) and a Hewlett Packard 1090L HPLC equipped with a diode array detector. Elution buffer A was: 20 mM Tris, 2 M ammonium sulfate, 20% (v/v) glycerol, pH 8.0 while elution buffer B was: 20 mM Tris, 20% (v/v) glycerol, pH 8.0. The column was equilibrated with 10% elution buffer B at a flow rate of 1.0 mL/min for a minimum of 20 minutes. The sample load was 5 μg and protein was detected by monitoring the UV absorption at 214 mu using Turbochrom 3 data acquisition software (PE Nelson, Inc). Following injection of the sample, the column was maintained at 10% buffer B for 1 minute followed by a linear gradient of from 10% to 62% buffer B in 20 minutes. The column was washed with 100% buffer B for 5 minutes and re-equilibrated with 10% buffer B for a minimum of 20 minutes between successive sample injections.

[0153] Antibody Binding Activity: IgE receptor binding inhibition assay (IE25:2) was carried out as described in Presta et al., supra, on samples diluted to 20 μg/mL and 30 μg/mL in assay diluent (phosphate buffered saline, 0.5%

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BSA, 0.05% polysorbate 20, 0.01 % Thimerosol). Each dilution was then assayed in triplicate and the results were multiplied by an appropriate dilution factor to yield an active concentration. The results from 6 assays were averaged. The assay measures the ability of rhuMAb E25 to competitively bind to IgE and thereby prevent IgE from binding to its high affinity receptor which is immobilized to an ELISA plate. The results are divided by the antibody concentration as determined by UV absorption spectroscopy and reported as a specific activity. Previous experiments have shown that this assay is stability indicating.

[0154] Particulate Assay: Reconstituted vials of lyophilized rhuMAb E25 were pooled to achieve a volume of approximately 7 mL. A count of the number of particles of size ranging from 2 to 80 μm present in 1 mL of sample was determined using a Hiac/Royco model 8000 counter. The counter was first washed with 1 mL of sample three times followed by the measurement of 1 mL of sample in triplicate. The instrument determines the number of particles per mL that are equal to or greater than 10 μm and the number of particles per mL that are equal to or greater than 25 μm.

[0155] The first step in the development of a formulation for the anti-IgE antibody was to determine a suitable buffer and pH for lyophilization and storage of the product. Antibody at a concentration of 5.0 mg/mL was formulated into 10 mM succinate buffers ranging from pH 5.0 to pH 6.5 and into sodium phosphate, potassium phosphate and histidine buffers at pH 7.0. **FIG. 9** shows increased antibody aggregate was observed in the higher pH formulations both before and after lyophilization. An exception was the histidine formulation at pH 7, where no increase in aggregate was observed upon storage at 2-8° C. **FIG. 10** shows rhuMAb E25 lyophilized in 5 mM histidine buffer at both pH 6 and pH 7 and stored for 1 year at 2-8° C., 25° C., and 40° C. At each assay time point and storage temperature the pH 6 formulation had less aggregate than the antibody formulated at pH 7. These results show histidine at pH 6 is a particularly useful buffer system for preventing aggregation of the antibody.

[0156] To facilitate screening of lyoprotectants, the anti-IgE antibody was formulated into sodium succinate at pH 5 with or without a lyoprotectant. Potential lyoprotectants, added at isotonic concentrations, were grouped into 3 categories:

[0157] (a) non-reducing monosaccharide (i.e. mannitol);

[0158] (b) reducing disaccharides (i.e. lactose and maltose); and

[0159] (c) non-reducing disaccharides (i.e. trehalose and sucrose).

[0160] Aggregation of the formulations following storage at 2-8° C. and 40° C. for one year is shown in **FIGS. 11 and 12**. With storage at 2-8° C., the monosaccharide formulation (mannitol) aggregated at a rate similar to the buffer control, while formulations containing the disaccharides were equally effective in controlling aggregation (**FIG. 11**). The results following storage at 40° C. where similar with the exception of the sucrose formulation which rapidly aggregated (which correlated with a browning of the freeze-dried cake (**FIG. 12**)). This was later shown to be caused by degradation of sucrose following storage at both acidic pH and high temperature.

[0161] Hydrophobic interaction chromatography of the antibody formulated in histidine buffer at pH 6 with lactose shows the antibody is altered following storage for 6 months at 40° C. (**FIG. 13**). The chromatography peaks are broadened and the retention time decreases. These changes are not observed with the buffer control and sucrose formulations stored under similar conditions as shown in **FIGS. 14 and 15**, respectively. Furthermore, isoelectric focusing showed an acidic shift in the pI of the antibody formulated in lactose and stored at 25° C. and 40° C. This indicates that reducing sugars are not suitable as lyoprotectants for the antibody.

[0162] Aggregation of lyophilized formulations of anti-IgE at a concentration of 20 mg/mL in 5 mM histidine buffer at pH 6 with various concentrations of sucrose and trehalose following storage for 12 weeks at 50° C. is shown in **FIG. 16**. Both sugars have a similar protective effect on aggregation when the sugar concentration is greater than 500 moles of sugar per mole of antibody. From these results, isotonic and hypertonic formulations of both sucrose and trehalose were identified for further development. The formulations are designed to be filled prior to lyophilization at a relatively low concentration of antibody and the lyophilized product is reconstituted with less volume than was filled with bacteriostatic water for injection (BWFI) comprising 0.9% benzyl alcohol. This allows the concentration of the antibody immediately prior to subcutaneous delivery and includes a preservative for a potential multi-use formulation while avoiding interactions between the protein and preservative upon long-term storage.

[0163] Isotonic formulation: Anti-IgE at 25 mg/mL formulated in 5 mM histidine buffer at pH 6 with 500 moles of sugar per mole antibody which equals a sugar concentration of 85 mM. This formulation is reconstituted with BWFI (0.9% benzyl alcohol) at a volume which is four times less than was filled. This results in a 100 mg/mL of antibody in 20 mM histidine at pH 6 with an isotonic sugar concentration of 340 mM.

[0164] Hypertonic formulation: Anti-IgE at 25 mg/mL formulated in 5 mM histidine buffer at pH 6 with 1000 moles of sugar per mole antibody which equals a sugar concentration of 161 mM. This formulation is reconstituted with BWFI (0.9% benzyl alcohol) at a volume which is four times less than was filled. This results in a 100 mg/mL of antibody in 20 mM histidine at pH 6 with a hypertonic sugar concentration of 644 mM.

[0165] Comparisons of the antibody aggregation following storage of the isotonic and hypertonic formulations for up to 36 weeks are shown in FIGS. **17** to **19**. No change in aggregation is observed in either the hypertonic or isotonic formulations with storage at 2-8° C. (**FIG. 17**). With storage at controlled room temperature (30° C.) increased aggregation is not observed in the hypertonic formulations while an increase in aggregate of from 1 to 2% occurs in the isotonic formulations (**FIG. 18**). Finally, following storage at 50° C. a minimal increase in aggregate is observed with the hypertonic formulations, a 4% increase in aggregate occurs with the isotonic trehalose formulation and a 12% increase in aggregate occurs with the isotonic sucrose formulation (**FIG. 19**). These results show the isotonic formulation contains the minimum amount of sugar necessary to maintain the stability of the antibody with storage at a temperature up to 30° C.

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[0166] The binding activity of the anti-IgE in the isotonic and hypertonic formulations was measured in an IgE receptor inhibition assay. It was discovered that the binding activity of the isotonic and hypertonic sucrose and trehalose formulations was essentially unchanged following storage at −70° C., 2-8° C., 30° C. and 50° C. for up to 36 weeks.

[0167] Lyophilized formulations of proteins are known to contain insoluble aggregates or particulates (Cleland et al., *Critical Reviews in Therapeutic Drug Carrier Systems,* 10 (4):307-377 (1993)). Accordingly, a particulate assay of antibody lyophilized at a concentration of 25 mg/mL in 5 mM histidine, pH 6 with the addition of 85 mM and 161 mM sucrose and trehalose was performed. Polysorbate 20 was added to the formulations at a concentration of 0.005%, 0.01%, and 0.02%. Samples were lyophilized and assayed following reconstitution to 100 mg/mL antibody in 20 mM histidine, pH 6 with 340 mM and 644 mM sugar. The polysorbate 20 concentration following reconstitution was 0.02%, 0.04%, and 0.08%.

[0168] Table 9 below shows the number of particles of size equal to or greater than 10 μm and equal to or greater than 25 μm from the isotonic and hypertonic sucrose and trehalose formulations. Polysorbate 20 was added to the formulations at concentrations of 0.005%, 0.01%, and 0.02% prior to lyophilization. The results show that the addition of Tween™ to the formulation significantly reduces the number of particles in each size range tested. The US Pharmacopeia (USP) specification for small volume injections are not more than 6,000 particles of greater than or equal to 10 μm and not more than 600 particles of greater than or equal to 25 μm per container (Cleland et al., supra). With the addition of polysorbate 20, both the hypertonic and isotonic formulations pass this specification.

TABLE 9

		Particles per mL	
Formulation	Polysorbate 20	≧10 μm	≧25 μM
Isotonic Sucrose	None	16,122	28
	0.005%	173	2
	0.01%	224	5
	0.02%	303	6
Hypertonic Sucrose	None	14,220	84
	0.005%	73	6
	0.01%	51	0
	0.02%		6
Isotonic Trehalose	None	33,407	24
	0.005%	569	4
	0.01%	991	16
	0.02%	605	9
Hypertonic Trehalose	None	24,967	28
	0.005%	310	11
	0.01%	209	6
	0.02%	344	6

[0169] One formulation developed for the anti-IgE antibody (i.e. 143 mg vial isotonic formulation of rhuMAb E25) which is considered to be useful for subcutaneous delivery of this antibody is shown in Table 10 below. A 10 cc vial is filled with 5.7 mL of rhuMAb E25 at a concentration of 25 mg/mL formulated in 5 mM histidine at pH 6.0 with 0.01% polysorbate 20. Sucrose is added as a lyoprotectant at a concentration of 85 mM which corresponds to a molar ratio of sugar to antibody of 500 to 1. The vial is lyophilized and reconstituted with 0.9% benzyl alcohol to one quarter of the volume of the fill or 1.2 mL. The final concentration of components in the formulation is increased four fold to 100 mg/mL rhuMAb E25 in 20 mM histidine at pH 6 with 0.04% polysorbate 20 and 340 mM sucrose (isotonic) and 0.9% benzyl alcohol. The formulation contains histidine buffer at pH 6 because of its demonstrated protective effect on antibody aggregation. Sucrose was added as the lyoprotectant because of previous use in the pharmaceutical industry. The concentration of sugar was chosen to result in an isotonic formulation upon reconstitution. Finally, polysorbate 20 is added to prevent the formation of insoluble aggregates.

TABLE 10

Pre-lyophilized Formulation (Fill 5.7 mL into 10 cc vial)	Reconstituted Formulation (1.2 mL 0.9% Benzyl Alcohol)
25 mg/mL rhuMAb E25	100 mg/mL rhuMAb E25
5 mM Histidine, pH 6.0	20 mM Histidine, pH 6.0
85 mM Sucrose	340 mM Sucrose
0.01% Polysorbate 20	0.04% Polysorbate 20
	0.9% Benzyl Alcohol

What is claimed is:

1. A stable isotonic reconstituted formulation comprising an antibody in an amount of at least about 50 mg/mL and a diluent, which reconstituted formulation has been prepared from a lyophilized mixture of an antibody and a lyoprotectant, wherein the antibody concentration in the reconstituted formulation is about 2-40 times greater than the antibody concentration in the mixture before lyophilization.

2. The formulation of claim 1 wherein the lyoprotectant is sucrose.

3. The formulation of claim 1 wherein the lyoprotectant is trehalose.

4. The formulation of claim 1 which further comprises a buffer.

5. The formulation of claim 4 wherein the buffer is histidine or succinate.

6. The formulation of claim 1 which further comprises a surfactant.

7. The formulation of claim 1 which is sterile.

8. A stable reconstituted formulation comprising an antibody in an amount of at least about 50 mg/mL and a diluent, which reconstituted formulation has been prepared from a lyophilized mixture of an antibody and a lyoprotectant, wherein the antibody concentration in the reconstituted formulation is about 2-40 times greater than the antibody concentration in the mixture before lyophilization.

9. The formulation of claim 8 wherein the antibody is an anti-IgE antibody.

10. The formulation of claim 8 wherein the antibody is an anti-HER2 antibody.

11. The formulation of claim 8 wherein the antibody is a full length humanized antibody.

12. A method for preparing a stable isotonic reconstituted formulation comprising reconstituting a lyophilized mixture of a protein and a lyoprotectant in a diluent such that the protein concentration in the reconstituted formulation is at least 50 mg/mL, wherein the protein concentration in the reconstituted formulation is about 2-40 times greater than the protein concentration in the mixture before lyophilization.

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13. The method of claim 12 wherein the lyoprotectant is sucrose.

14. The method of claim 12 wherein the lyoprotectant is trehalose.

15. The method of claim 12 wherein the lyophilized mixture further comprises a bulking agent.

16. The method of claim 12 wherein the protein is a monoclonal antibody.

17. A method for preparing a formulation comprising the steps of:

(a) lyophilizing a mixture of an antibody and a lyoprotectant; and

(b) reconstituting the lyophilized mixture of step (a) in a diluent such that the reconstituted formulation is isotonic and stable and has a protein concentration of at least about 50 mg/mL.

18. The method of claim 17 wherein the protein concentration in the reconstituted formulation is from about 80 mg/mL to about 300 mg/mL.

19. The method of claim 17 wherein the protein concentration in the reconstituted formulation is about 2-40 times greater than the protein concentration in the mixture before lyophilization.

20. The method of claim 17 wherein lyophilization is performed at a shelf temperature maintained at about 15-30° C. throughout the entire lyophilization process.

21. An article of manufacture comprising:

(a) a container which holds a lyophilized mixture of an antibody and a lyoprotectant; and

(b) instructions for reconstituting the lyophilized mixture with a diluent to a protein concentration in the reconstituted formulation of at least about 50 mg/mL.

22. The article of manufacture of claim 21 wherein the protein concentration in the reconstituted formulation is about 2-40 times greater than the protein concentration in the mixture before lyophilization.

23. The article of manufacture of claim 21 further comprising a second container which holds a diluent.

24. The article of manufacture of claim 23 wherein the diluent is bacteriostatic water for injection (BWFI) comprising an aromatic alcohol.

25. A formulation comprising a lyophilized mixture of a lyoprotectant and an antibody, wherein the molar ratio of lyoprotectant:antibody is about 100-1500 mole lyoprotectant: 1 mole antibody.

26. A formulation comprising a lyophilized mixture of a lyoprotectant and an antibody, wherein the molar ratio of lyoprotectant to antibody is about 100-600 mole lyoprotectant: 1 mole antibody.

27. The formulation of claim 26 wherein the antibody is a humanized antibody.

28. The formulation of claim 26 wherein the antibody is an antibody fragment which binds antigen.

29. The formulation of claim 26 wherein the antibody is an anti-HER2 antibody.

30. The formulation of claim 26 wherein the antibody is an anti-IgE antibody.

31. The formulation of claim 26 wherein the lyoprotectant is sucrose.

32. The formulation of claim 26 wherein the lyoprotectant is trehalose.

33. The formulation of claim 26 which further comprises a buffer.

34. The formulation of claim 33 wherein the buffer is histidine or succinate.

35. The formulation of claim 26 further comprising a surfactant.

36. The formulation of claim 8 wherein the diluent is selected from the group consisting of sterile water, bacteriostatic water for injection (BWFI), a pH buffered solution, sterile saline solution, Ringer's solution or dextrose solution.

37. The article of manufacture of claim 23 wherein the diluent is selected from the group consisting of sterile water, bacteriostatic water for injection (BWFI), a pH buffered solution, sterile saline solution, Ringer's solution or dextrose solution.

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

(10) **Patent No.: US 6,355,245 B1**
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(54) **C5-SPECIFIC ANTIBODIES FOR THE TREATMENT OF INFLAMMATORY DISEASES**

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(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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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

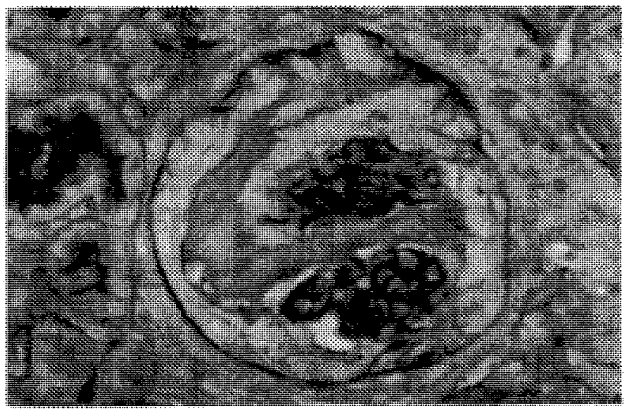


FIG. 1B



FIG. 1C

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FIG. 2A



FIG. 2B



FIG. 2C

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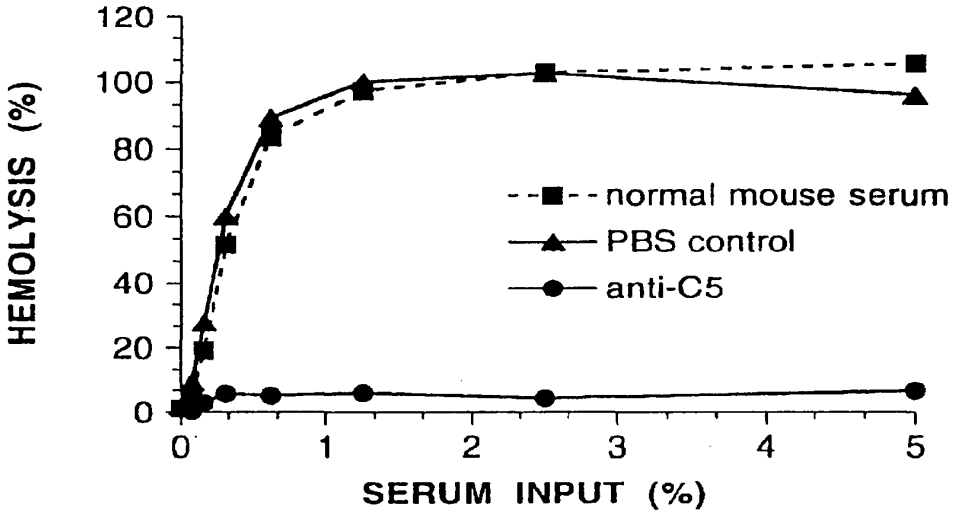


FIG.3

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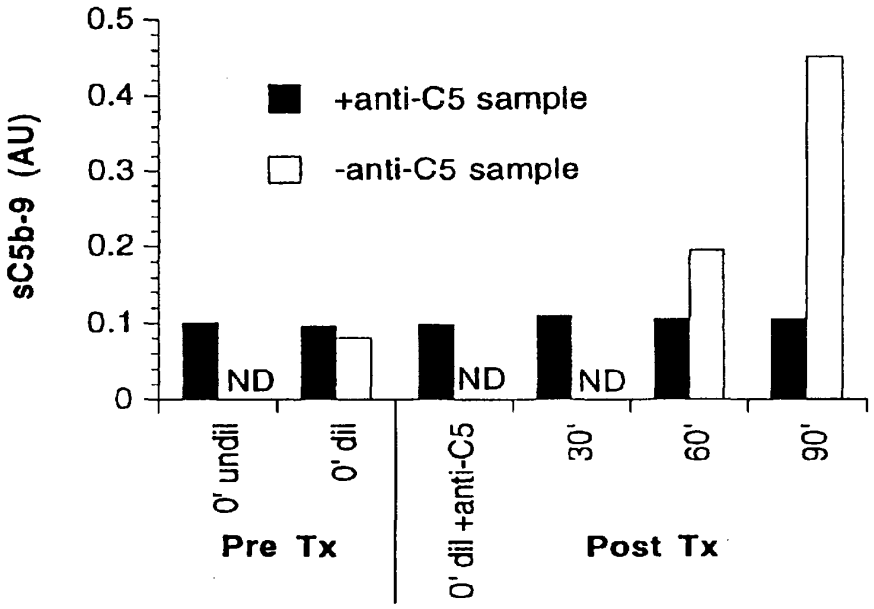


FIG.4

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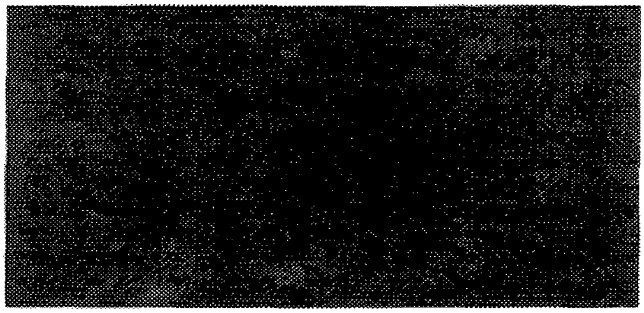


FIG.5A



FIG.5B



FIG.5C

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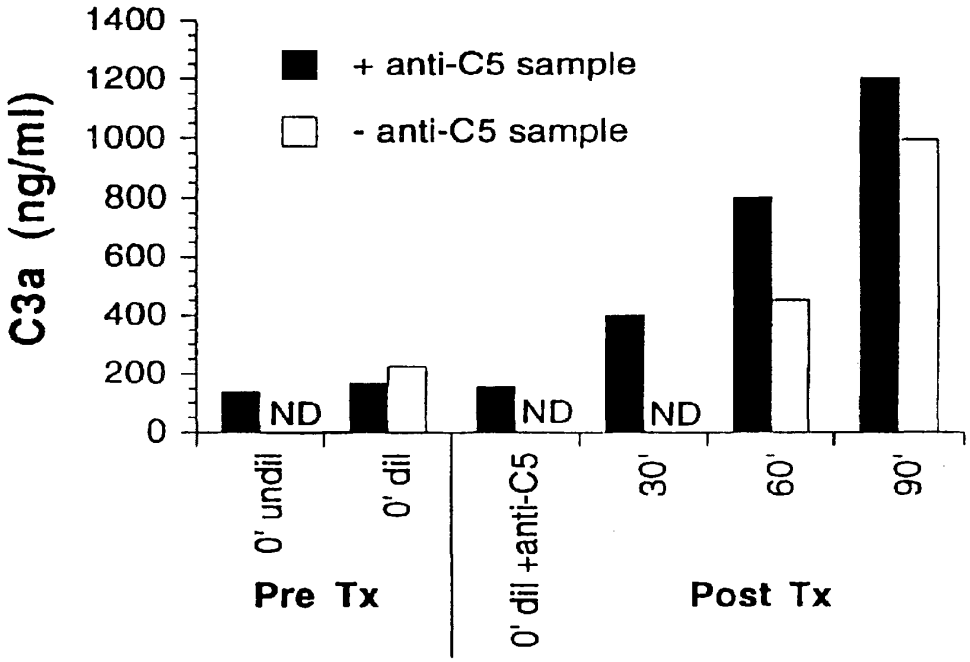


FIG.6

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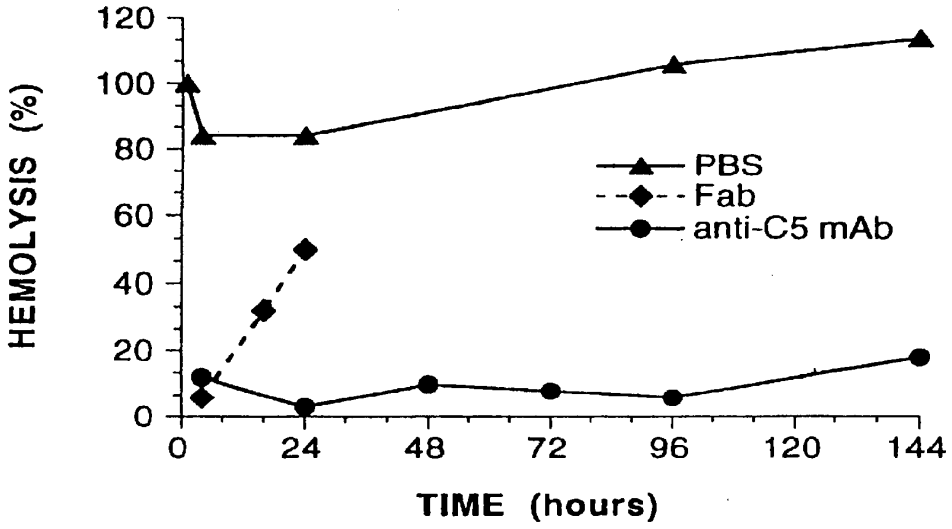


FIG.7A

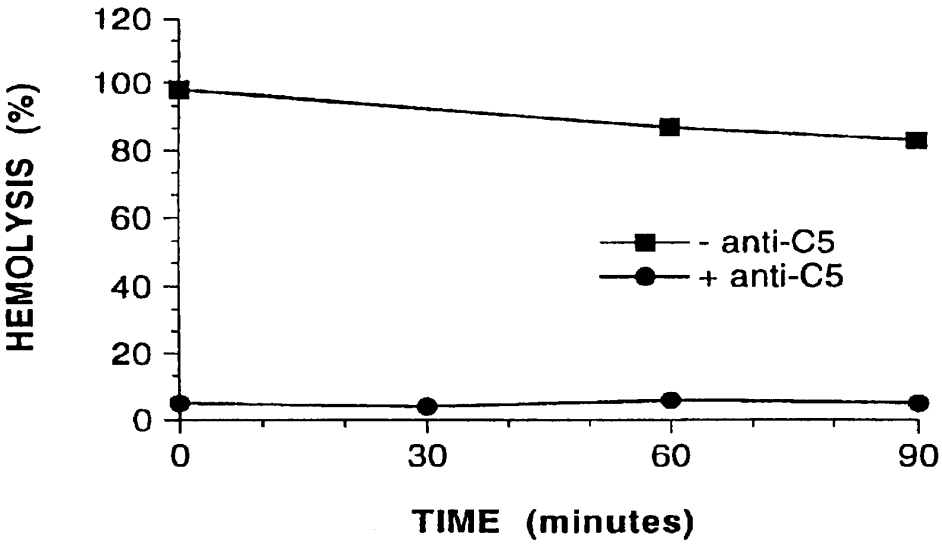


FIG.7B

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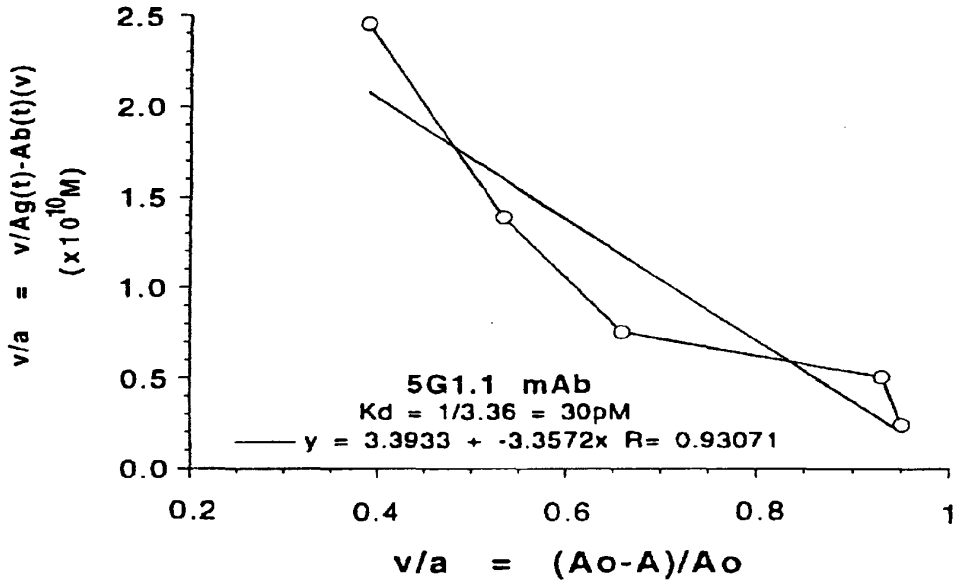

2.5
2.0
1.5
1.0
0.5
0.0
0.2
0.4
0.6
0.8
1
v/a = v/Ag(t)-Ab(t)(v)
(x10^10M)
5G1.1 mAb
Kd = 1/3.36 = 30pM
y = 3.3933 + -3.3572x R= 0.93071
v/a = (Ao-A)/Ao

FIG.8

350

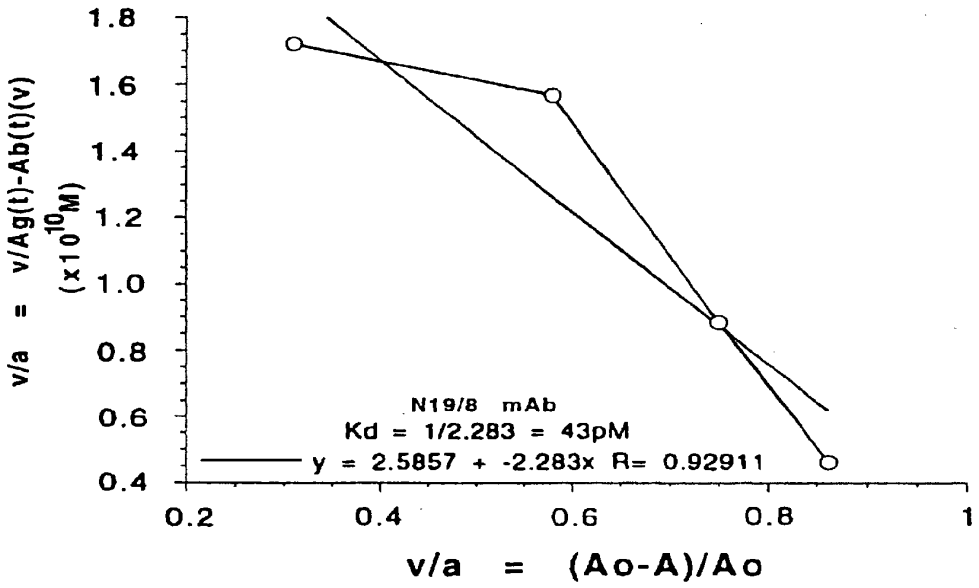


FIG.9

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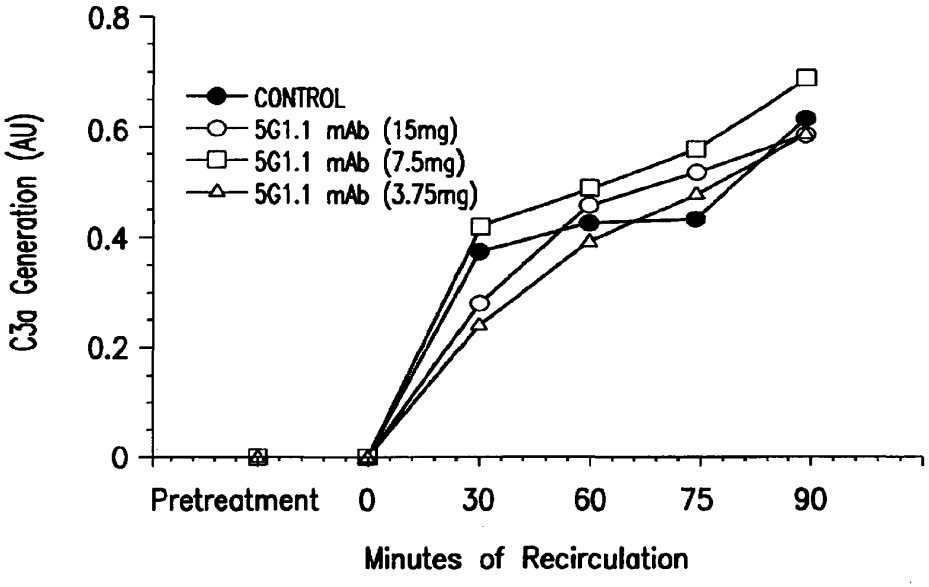


FIG.10

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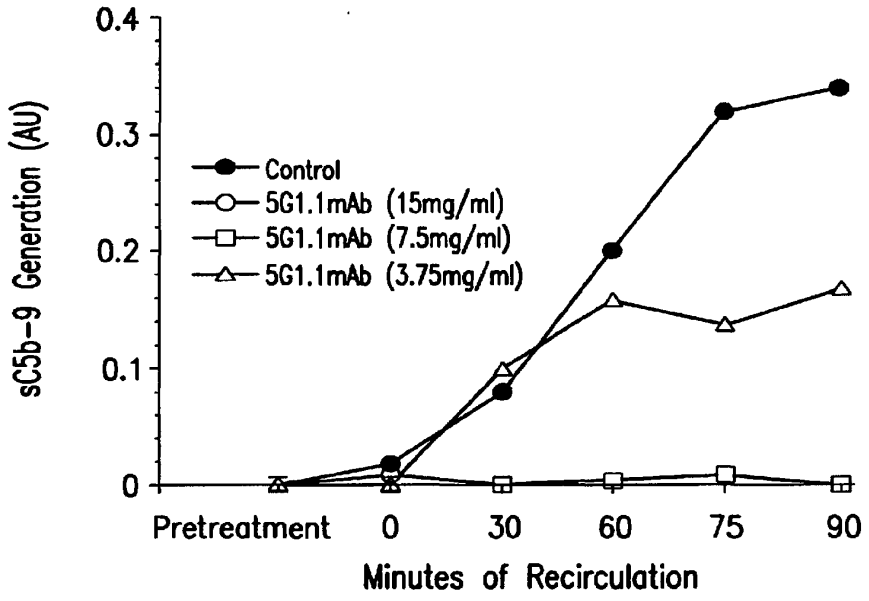


FIG. 11

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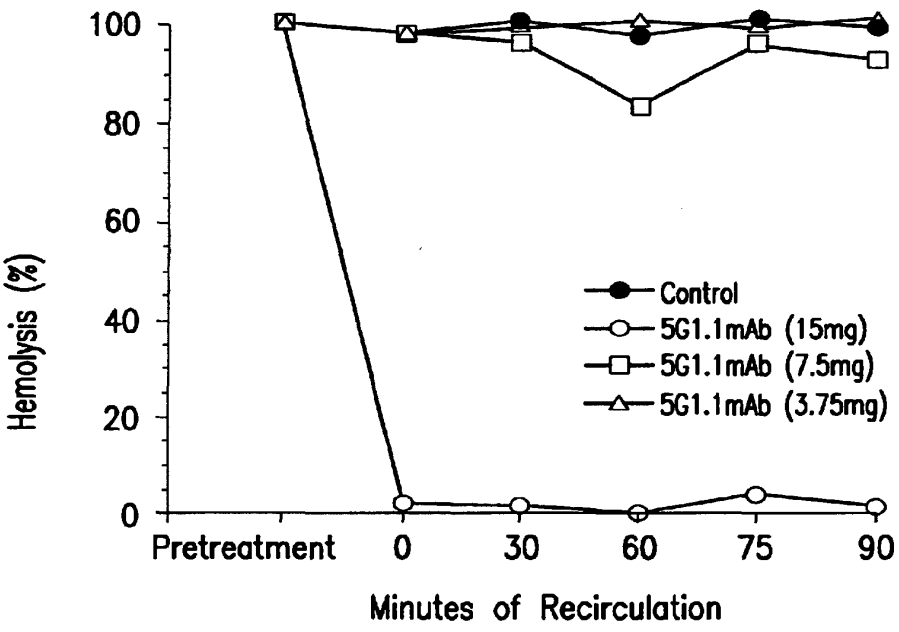


FIG. 12

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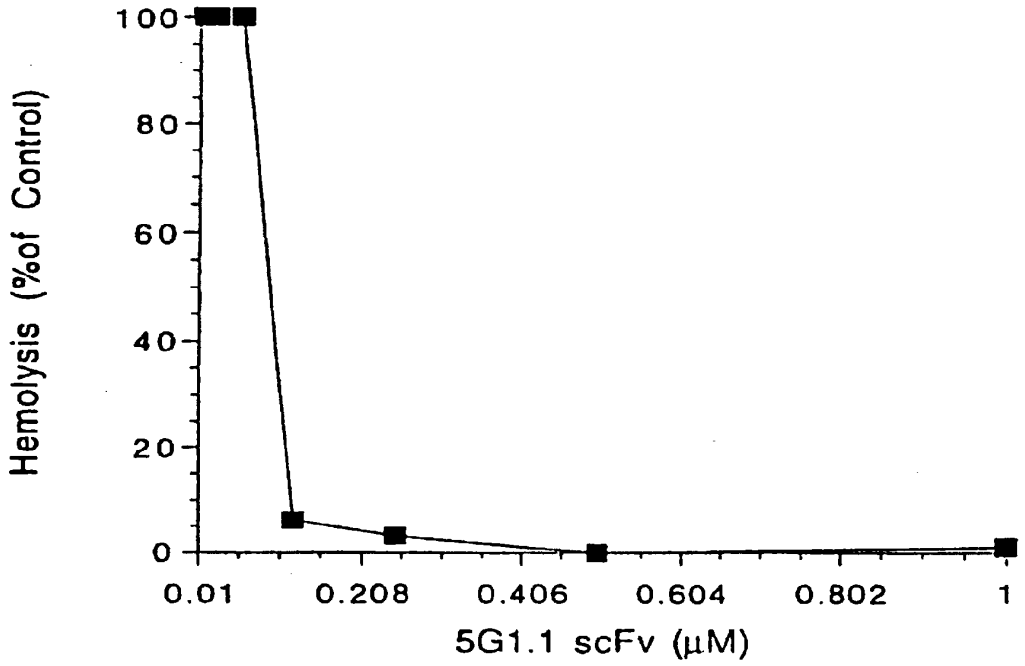


FIG.13

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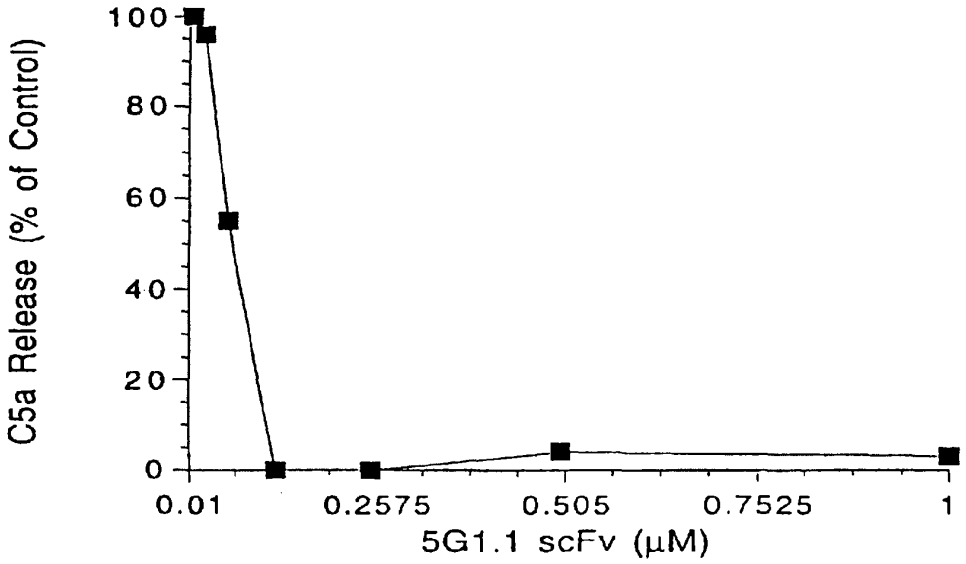


FIG. 14

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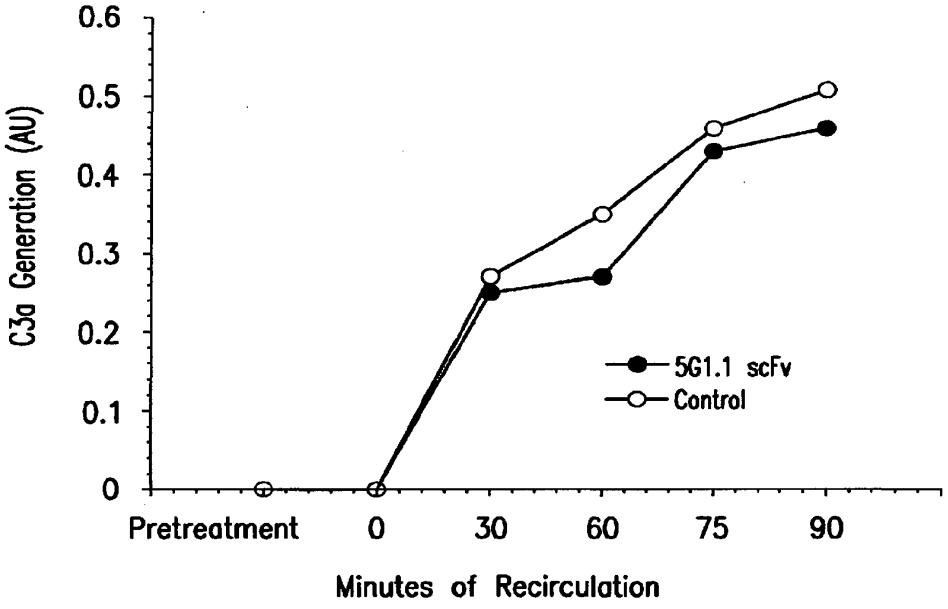


FIG. 15

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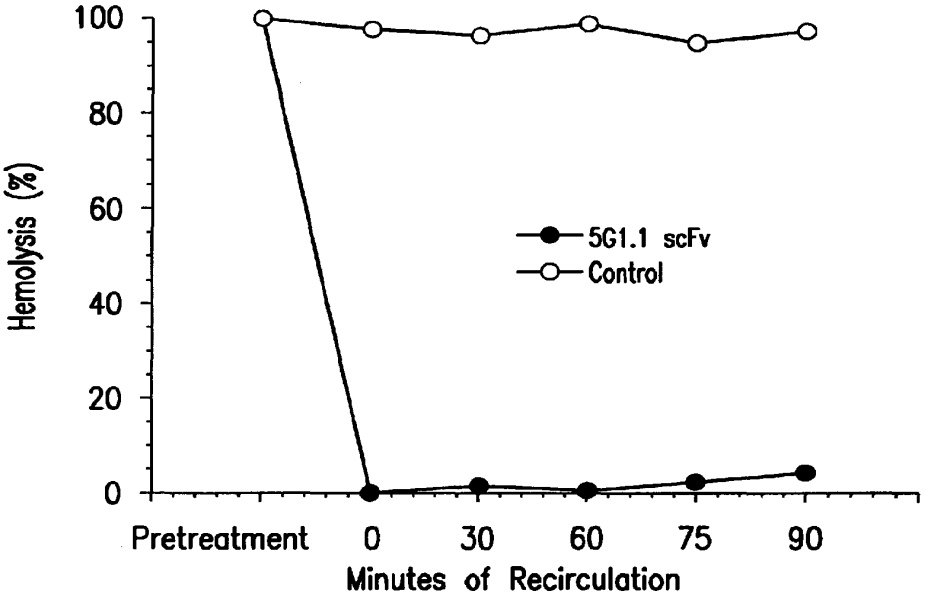


FIG. 16

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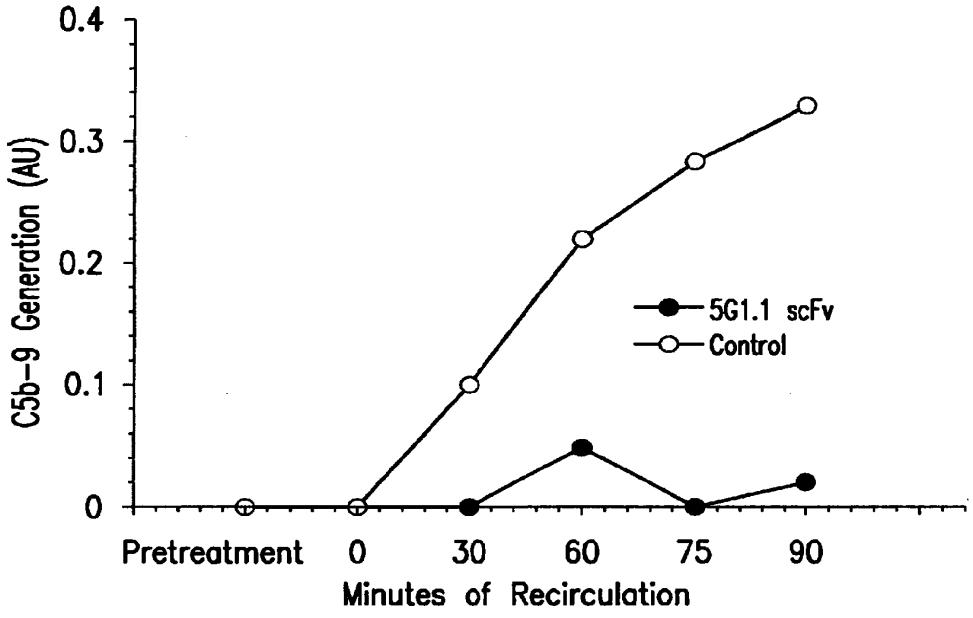


FIG. 17

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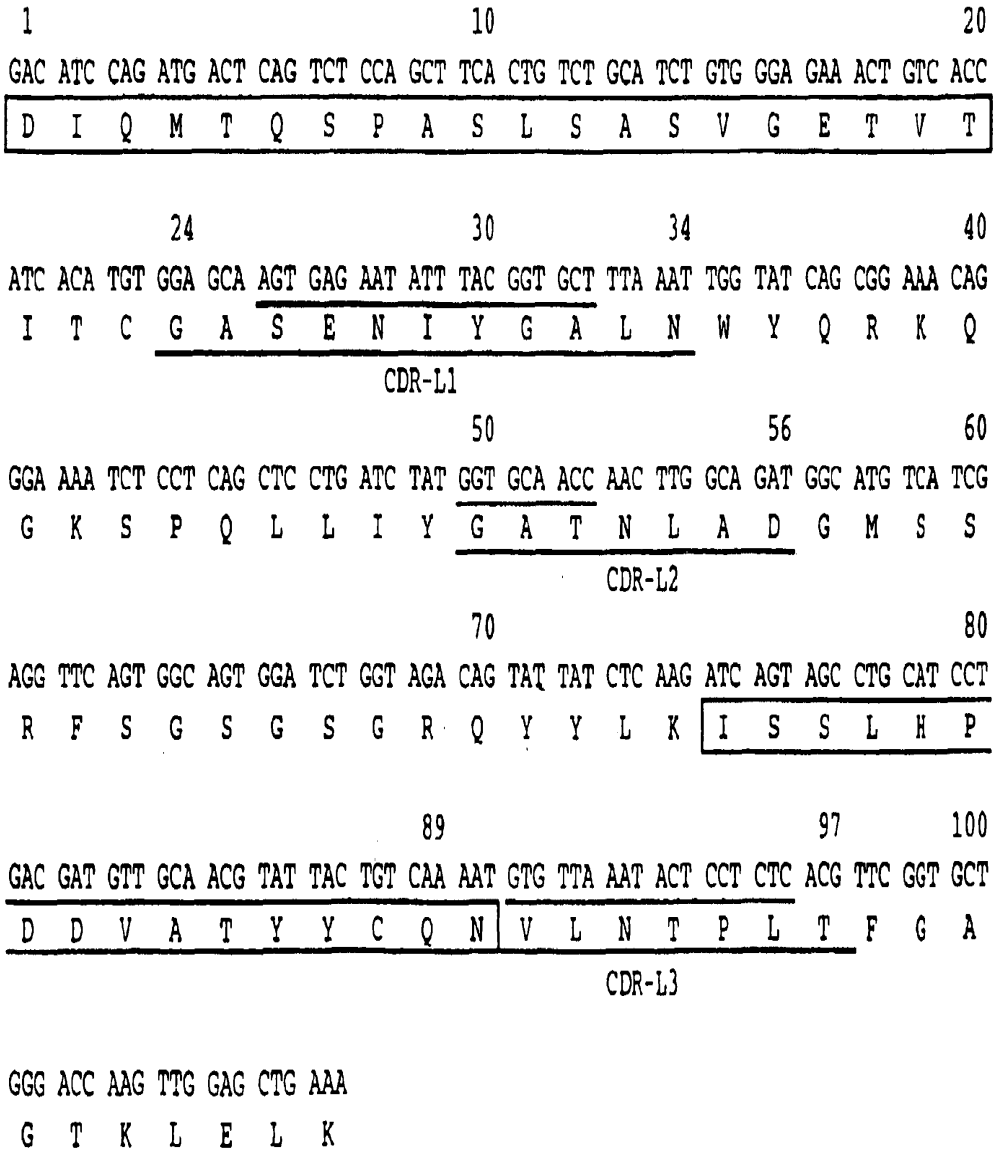


FIG.18

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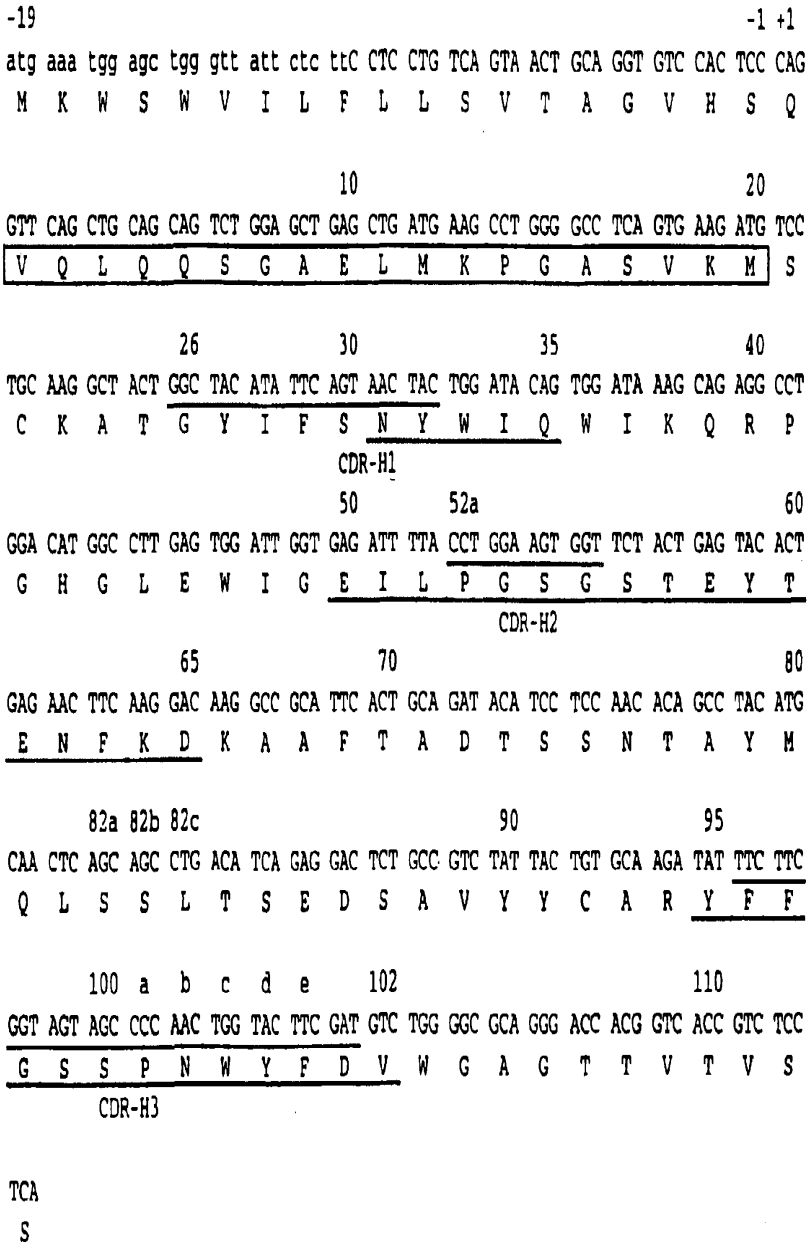


FIG.19

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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.

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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)

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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.

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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

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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

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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

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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.

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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.

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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

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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

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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-

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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 1 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

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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.

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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-

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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.

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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.

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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. 1B) 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. 1C) were essentially indistinguishable from the glomeruli of the normal healthy kidneys of the uninduced untreated mice (FIG. 1A).

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. 1B, 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. 1A is approximately 100μ in diameter; the inflamed glomerulus of the GN-induced, PBS treated mouse shown in FIG. 1B is approximately 175μ in diameter; the non-inflamed glomerulus of the GN-induced, anti-C5-treated mouse shown in FIG. 1C 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. 2B) and the anti-C5-treated, GN-induced mice (FIG. 2C), but not in the untreated uninduced controls (FIG. 2A). 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. 2B and FIG. 2C.

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

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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. **5B**) and the anti-C5 antibody-treated (FIG. **5C**) 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. **5A**).

Note that the print shown in FIG. **5A** was overexposed compared to those of FIG. **5B** and FIG. **5C** 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. **5B** and FIG. **5C**.

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

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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.

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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).

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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,

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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

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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 (imm21) 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 BamH1 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

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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 $O.D._{550}$ 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.

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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

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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

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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

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(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

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
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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

AAACACAGGC ACAGTACTGA CAAACCCATA CACCTCCTCT GAAATACCCA 1450

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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CTCTTAAAAT AGAAAATGTC AAGTCAGTTA AGCAGGAAGT GGACTAACTG	1750
ACGCAGCTGG CCGTGCGACA TCCTCTTTTA ATTAGTTGCT AGGCAACGCC	1800
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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GCCCATCCCG CCCCTAACTC CGCCCAGTTC CGCCCATTCT CCGCCCCATG	2150
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GAGCTATTCC AGAAGTAGTG AGGAGGCTTT TTTGGAGGCC TAGGCTTTTG	2250
CAAAAAGGAG CTCCCAGCAA AAGGCCAGGA ACCGTAAAAA GGCCGCGTTG	2300
CTGGCGTTTT TCCATAGGCT CCGCCCCCCT GACGAGCATC ACAAAAATCG	2350
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CGTTTCCCCC TGGAAGCTCC CTCGTGCGCT CTCCTGTTCC GACCCTGCCG	2450
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TCAATGCTCA CGCTGTAGGT ATCTCAGTTC GGTGTAGGTC GTTCGCTCCA	2550
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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
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AGCGATCTGT CTATTTCGTT CATCCATAGT TGCCTGACTC CCCGTCGTGT	3150
AGATAACTAC GATACGGGAG GGCTTACCAT CTGGCCCCAG TGCTGCAATG	3200
ATACCGCGAG ACCCACGCTC ACCGGCTCCA GATTTATCAG CAATAAACCA	3250
GCCAGCCGGA AGGGCCGAGC GCAGAAGTGG TCCTGCAACT TTATCCGCCT	3300
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GTTAATAGTT TGCGCAACGT TGTTGCCATT GCTACAGGCA TCGTGGTGTC	3400
ACGCTCGTCG TTTGGTATGG CTTCATTCAG CTCCGGTTCC CAACGATCAA	3450
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GGTTATGGCA GCACTGCATA ATTCTCTTAC TGTCATGCCA TCCGTAAGAT	3600
GCTTTTCTGT GACTGGTGAG TACTCAACCA AGTCATTCTG AGAATAGTGT	3650

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GCCACATAGC AGAACTTTAA AAGTGCTCAT CATTGGAAAA CGTTCTTCGG 3750

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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

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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

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(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
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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
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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
```


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```
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

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(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
```


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```
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
```


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```
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)

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(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
```


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```
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
```


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```
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
```


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```
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
```


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```
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
```


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```
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
```


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```
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
```


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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 S05/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

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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

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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)

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(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
```


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```
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
```


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```
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)

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(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
```


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```
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
```


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```
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
```


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-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
```


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-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
```


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```
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
```


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```
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
```


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```
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
```


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-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

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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.

* * * * *

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US006171586B1

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

(10) **Patent No.: US 6,171,586 B1**
(45) **Date of Patent: Jan. 9, 2001**

(54) **ANTIBODY FORMULATION**

(75) Inventors: **Xanthe M. Lam**, San Francisco; **James Q. Oeswein**, Moss Beach, both of CA (US); **Boonsri Ongpipattanakul**, Bangkok (TH); **Zahra Shahrokh**, San Francisco; **Sharon X. Wang**, San Mateo, both of CA (US); **Robert P. Weissburg**, Greenville, DE (US); **Rita L. Wong**, San Mateo, CA (US)

(73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)

(*) Notice: Under 35 U.S.C. 154(b), the term of this patent shall be extended for 0 days.

(21) Appl. No.: **09/097,171**

(22) Filed: **Jun. 12, 1998**

Related U.S. Application Data

(60) Provisional application No. 60/053,087, filed on Jun. 13, 1997.

(51) **Int. Cl.**[7] **A61K 39/395**; C12P 21/08

(52) **U.S. Cl.** **424/130.1**; 424/152.1; 424/141.1; 424/154.1; 424/173.1; 530/388.75

(58) **Field of Search** 424/130.1, 152.1, 424/136.1, 141.1, 154.1, 173.1, 133.1; 530/388.75

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Primary Examiner—Patrick Nolan
Assistant Examiner—Marianne DiBrino
(74) *Attorney, Agent, or Firm*—Lee K. Tan; Wendy M. Lee

(57) **ABSTRACT**

A stable aqueous pharmaceutical formulation comprising a therapeutically effective amount of an antibody not subjected to prior lyophilization, a buffer maintaining the pH in the range from about 4.5 to about 6.0, a surfactant and a polyol is described, along with uses for such a formulation.

29 Claims, 25 Drawing Sheets

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of the letter / report / expert opinion / plaint / defense
dated 09.08.2017
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TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDK
THTCPPCPAPELLGGRMKQLEDKVEELLSKNYHLENEVARLKKLVGER

FIG. 1A

DIQMTQSPSSLSASVGDRVTITCRASQDINNYLNWYQQKPGKAPKLLIYYTSTLHSGVP
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FIG. 1B

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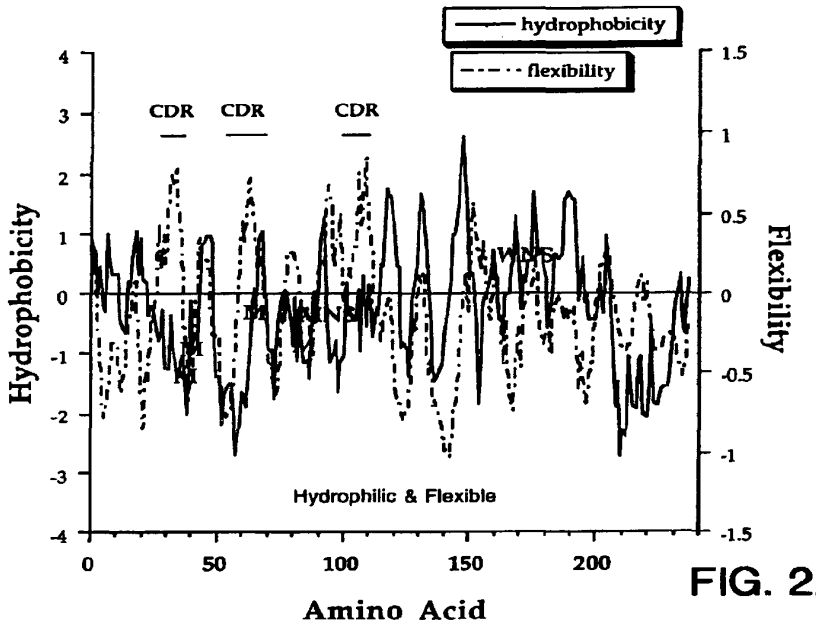


FIG. 2A

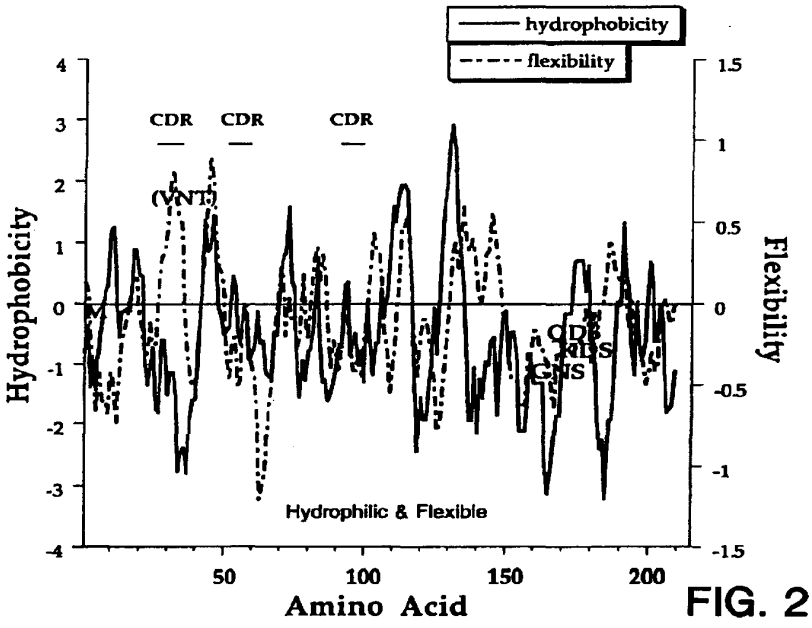


FIG. 2B

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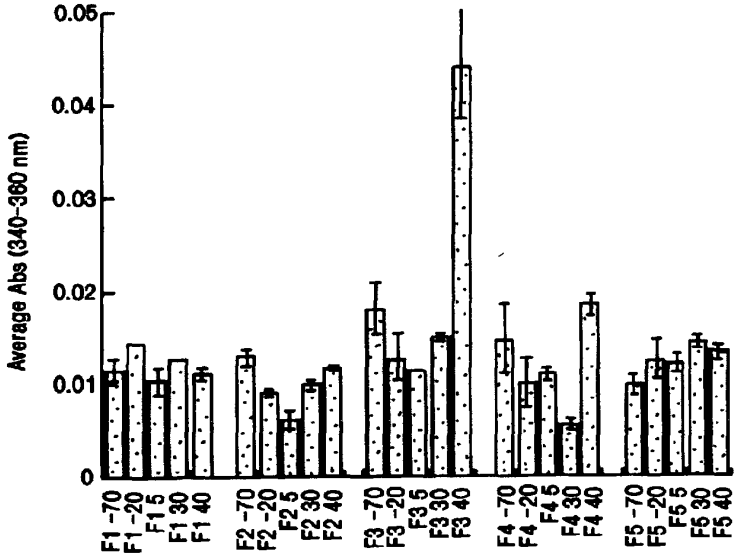


FIG. 3A

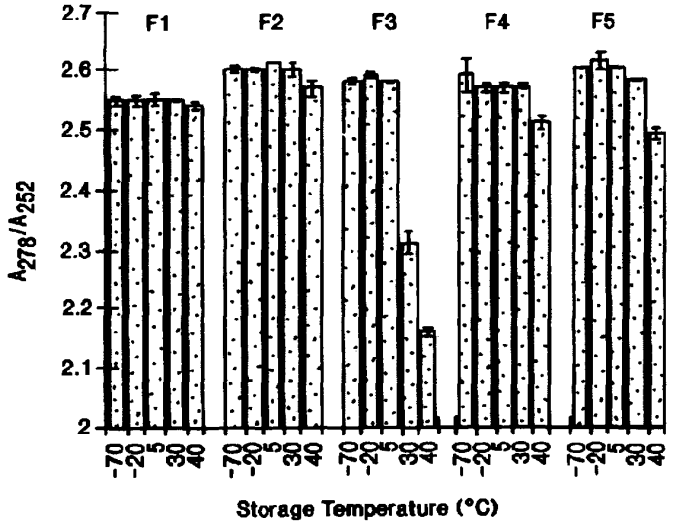


FIG. 3B

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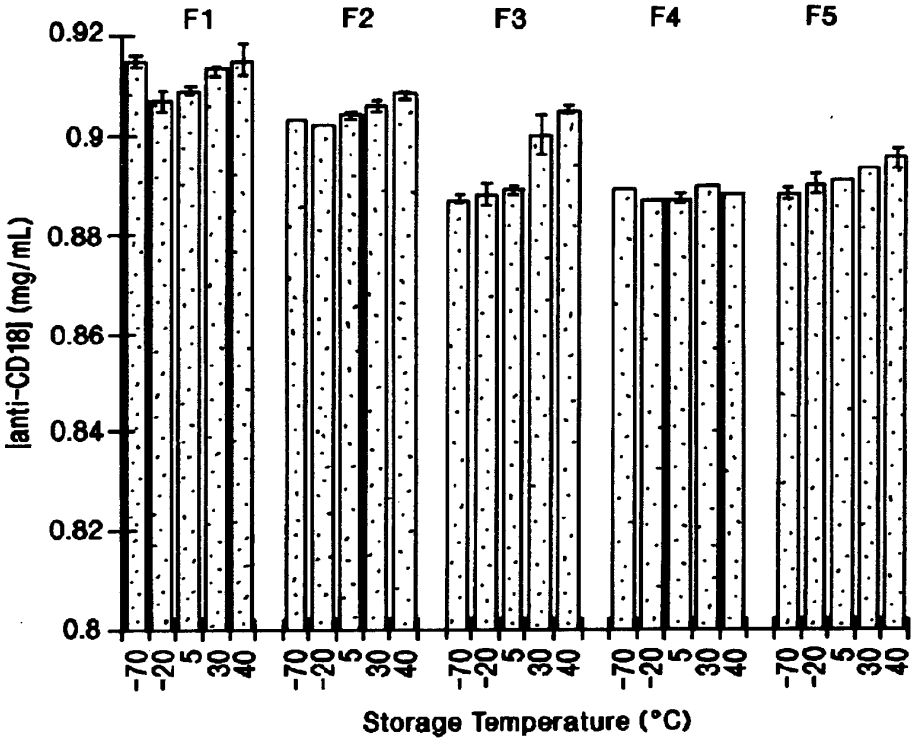


FIG. 4

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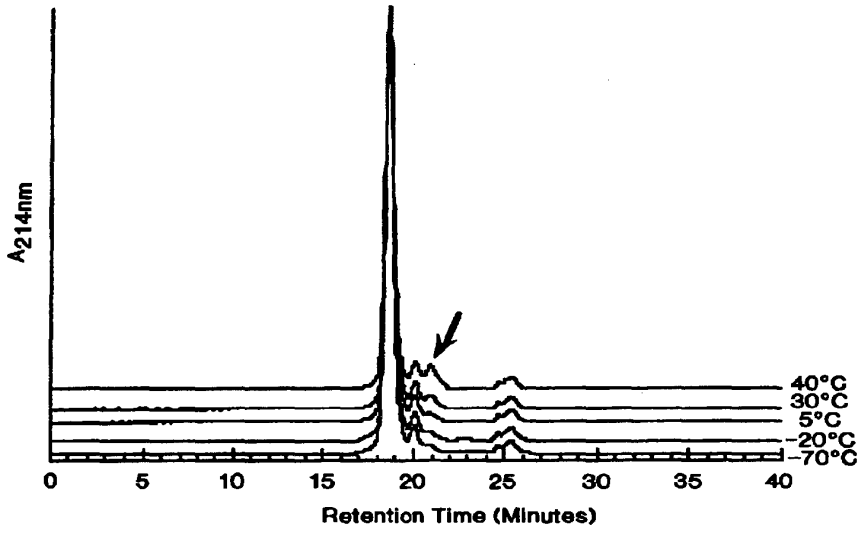


FIG. 5A

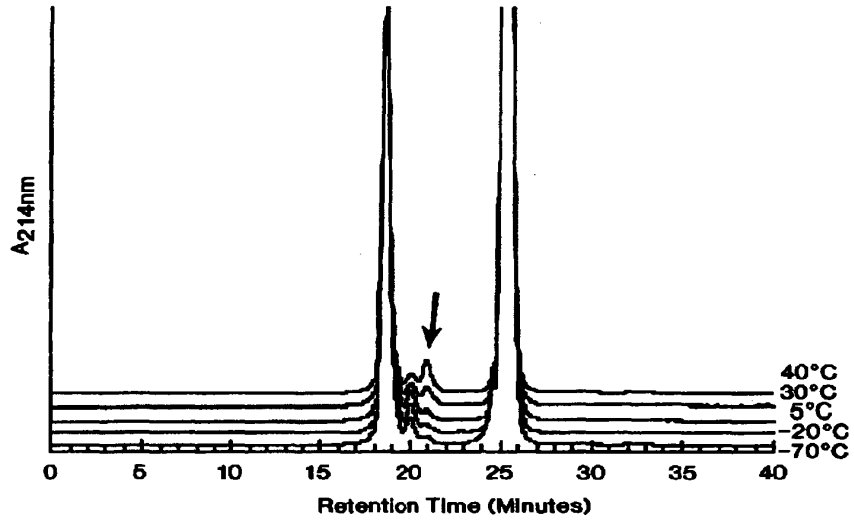


FIG. 5B

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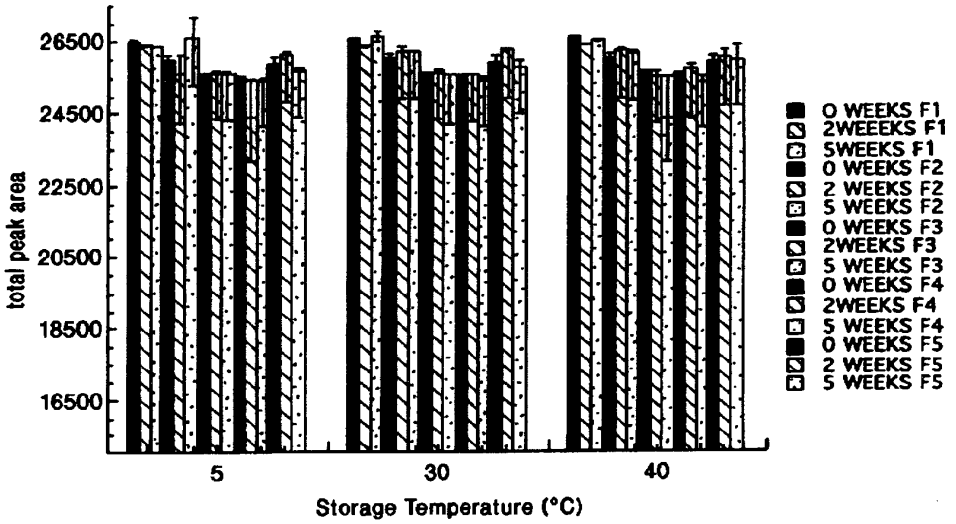


FIG. 6A

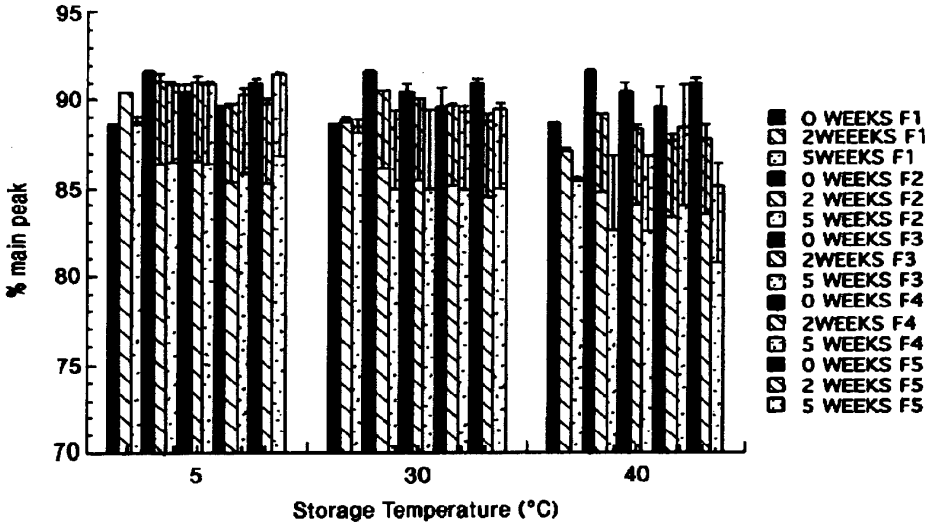


FIG. 6B

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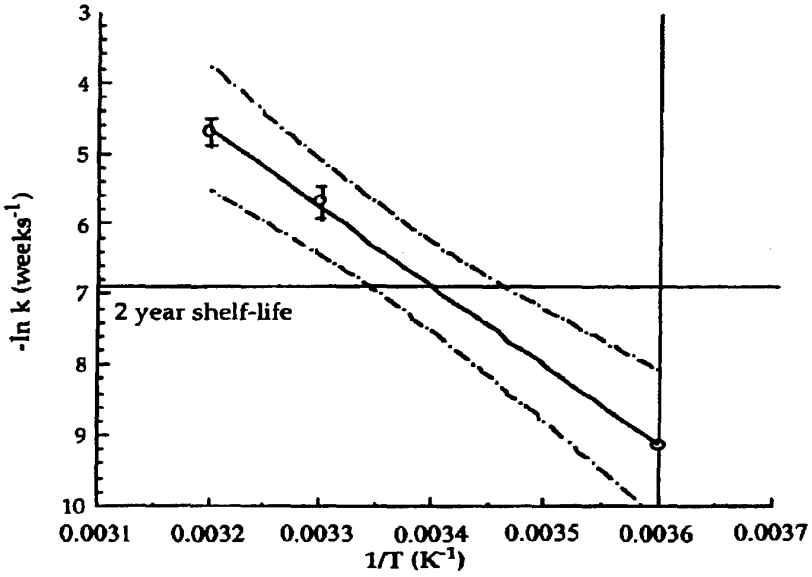


FIG. 7

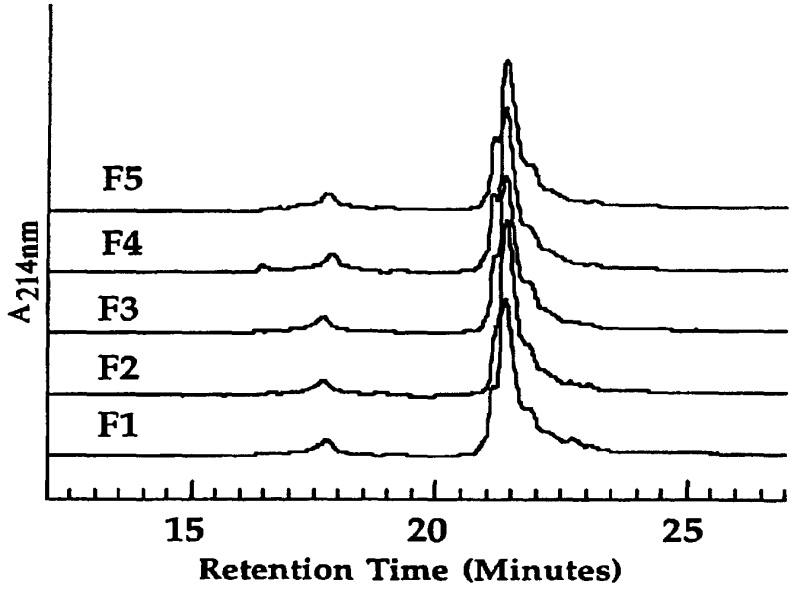


FIG. 8

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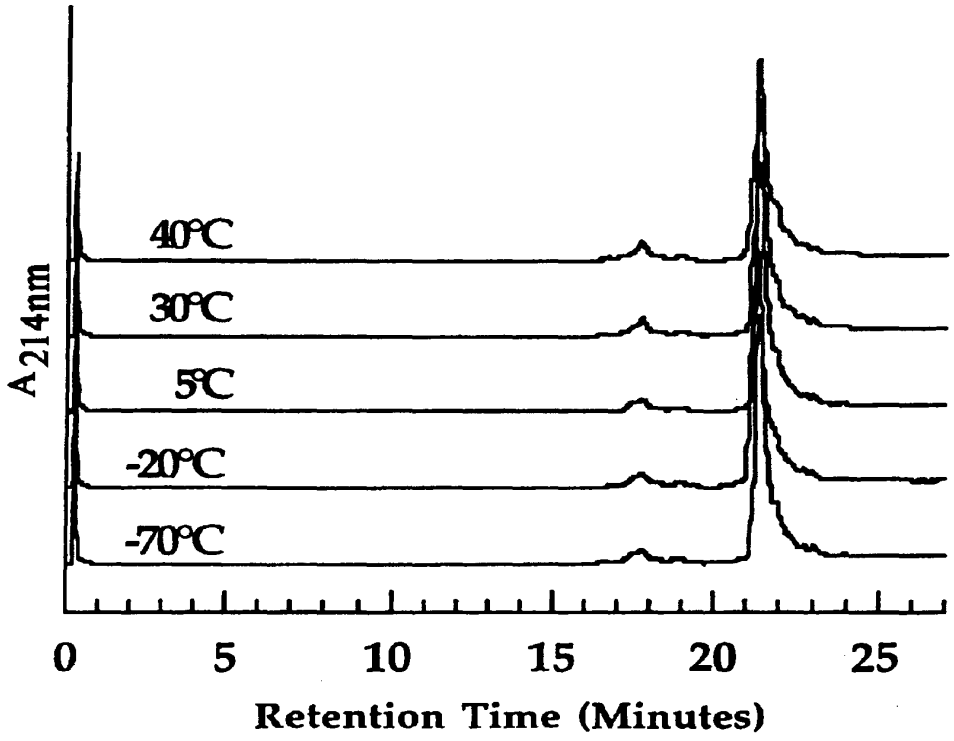


FIG. 9

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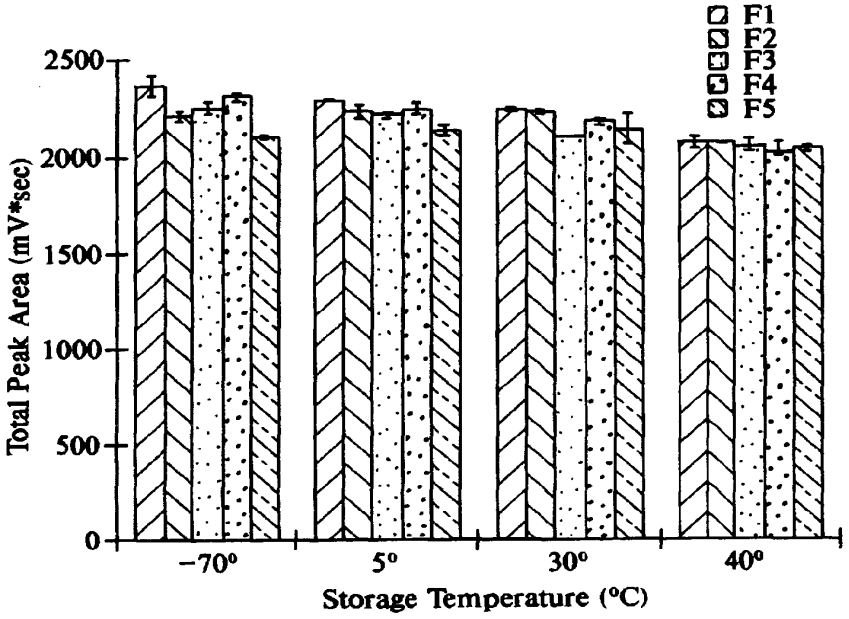


FIG. 10

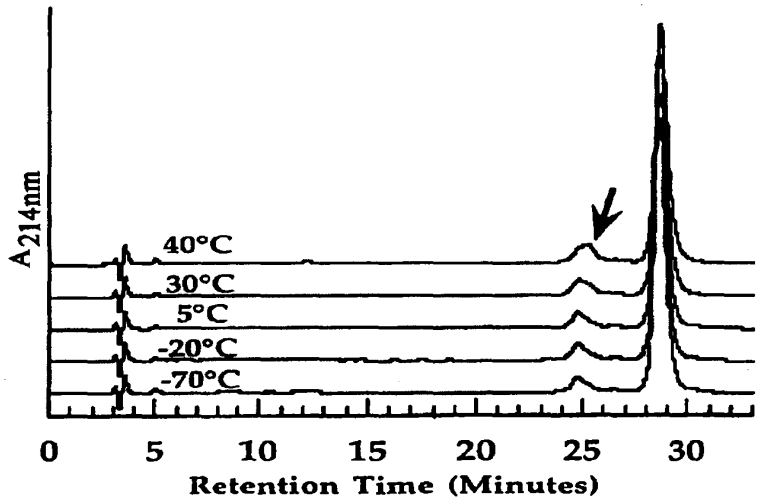


FIG. 11

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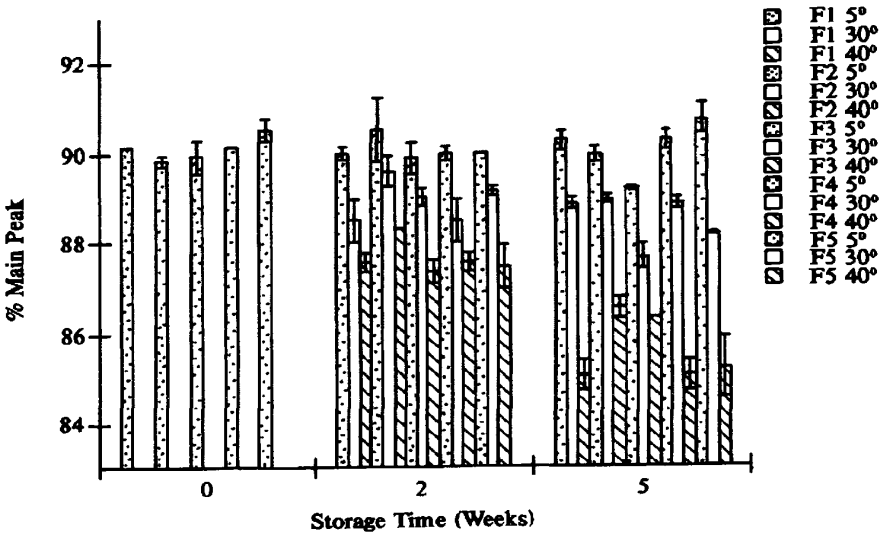


FIG. 12

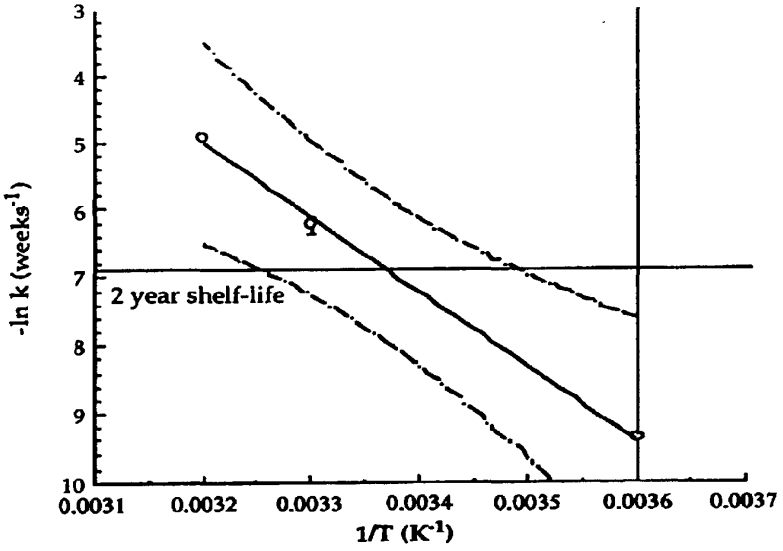


FIG. 13

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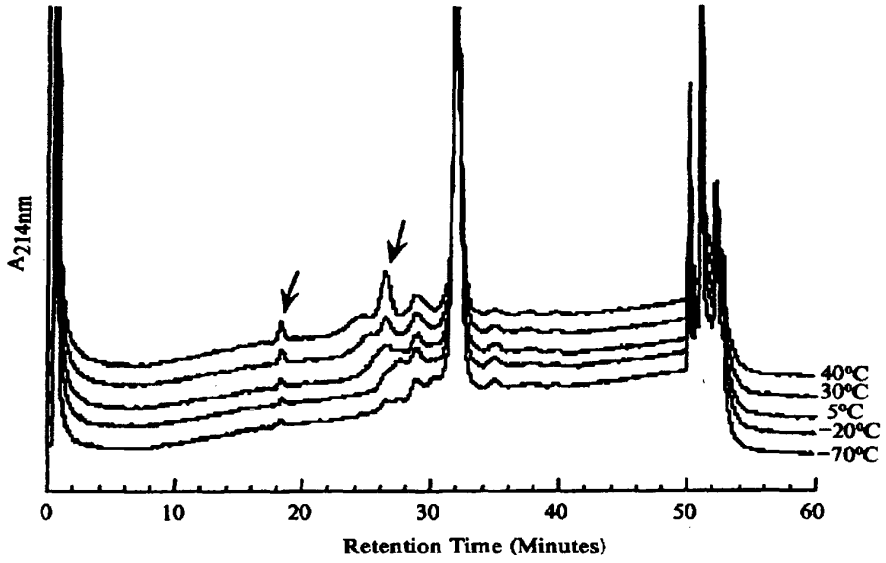


FIG. 14A

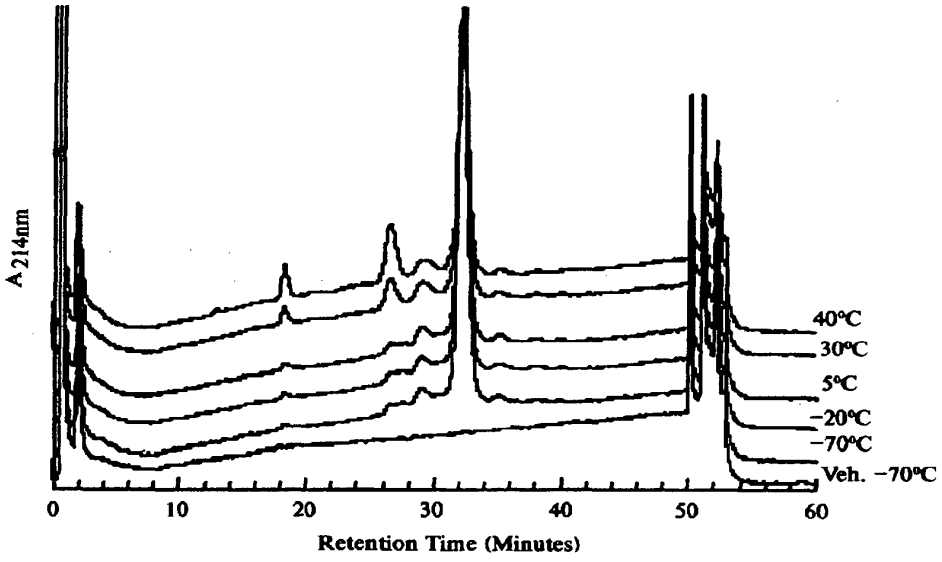


FIG. 14B

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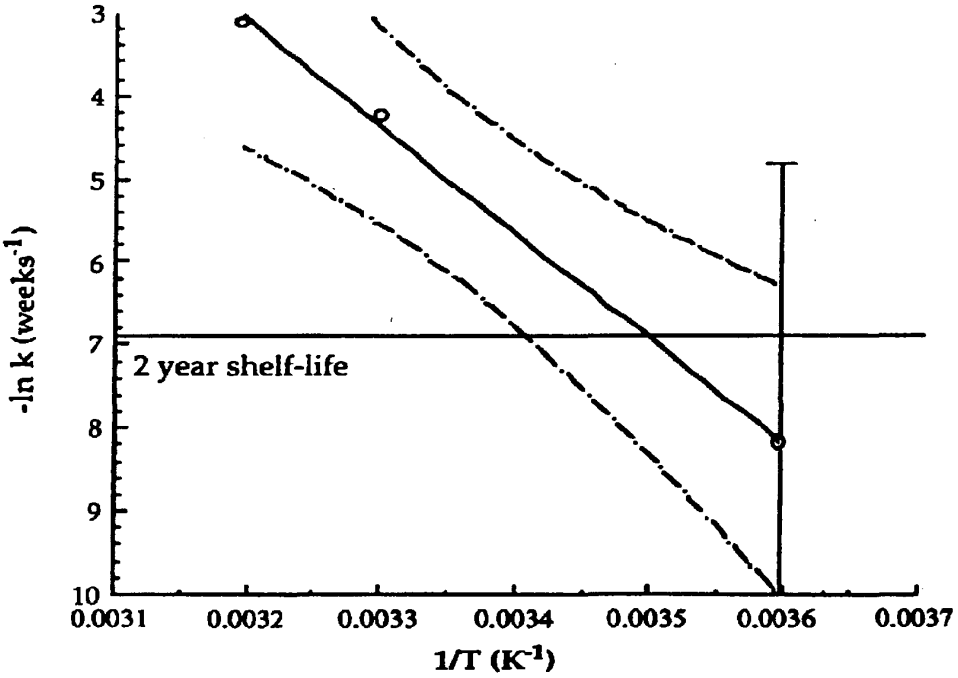


FIG. 15

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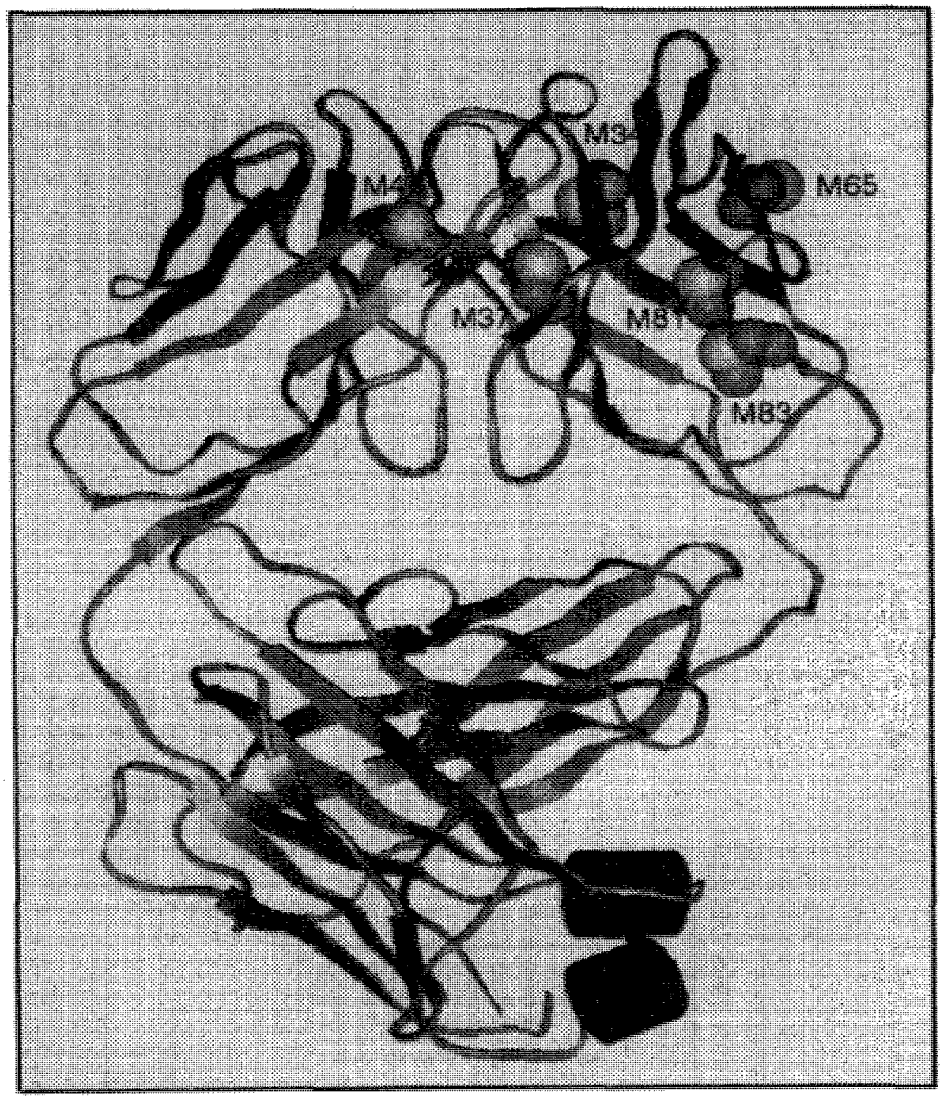


FIG. 16

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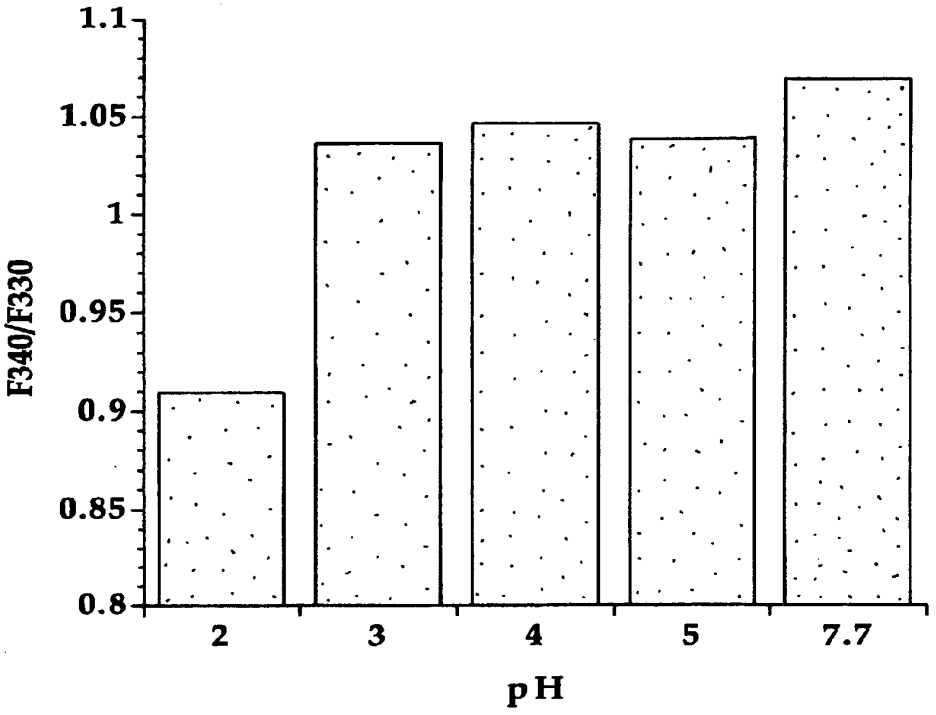


FIG. 17

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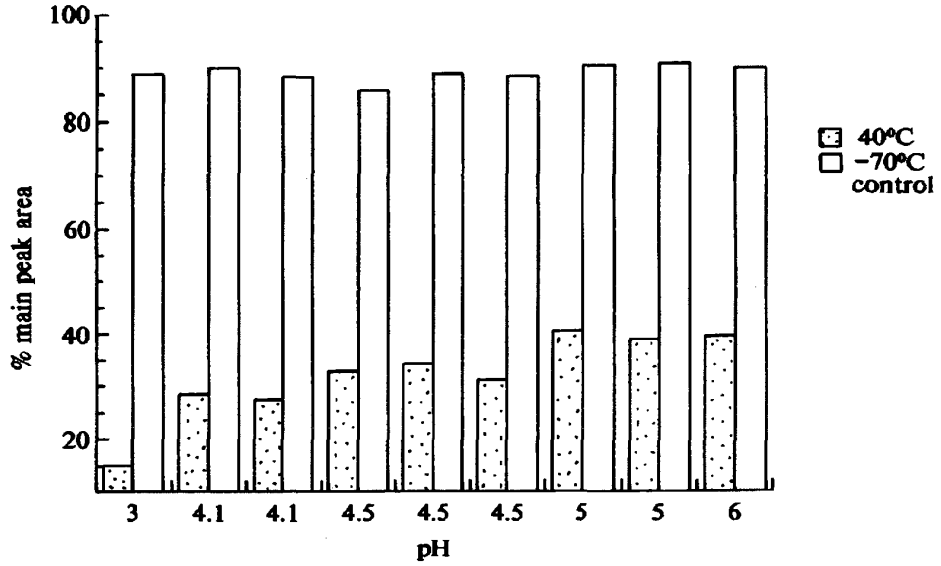


FIG. 18A

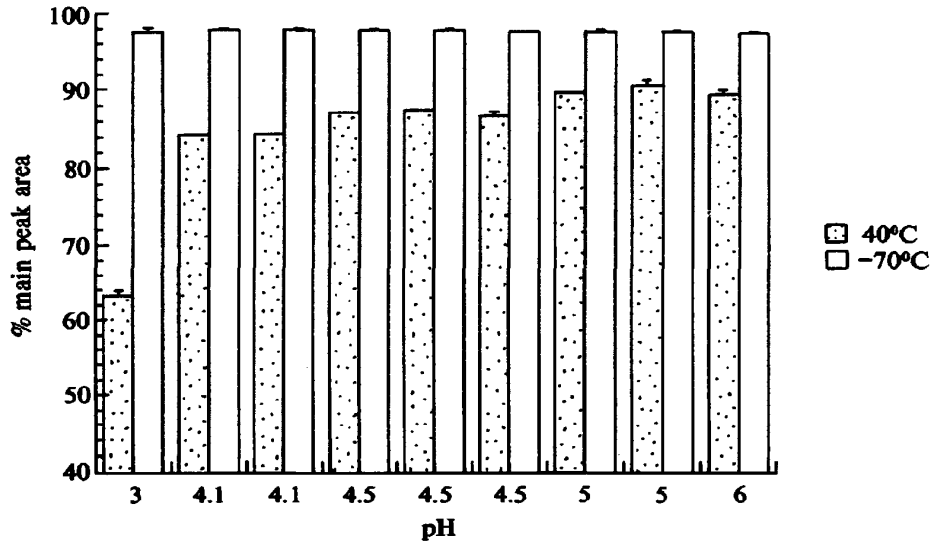


FIG. 18B

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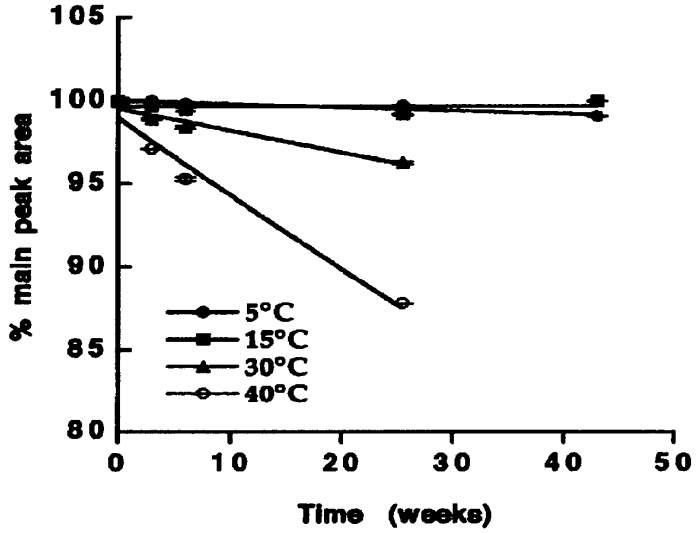


FIG. 19A

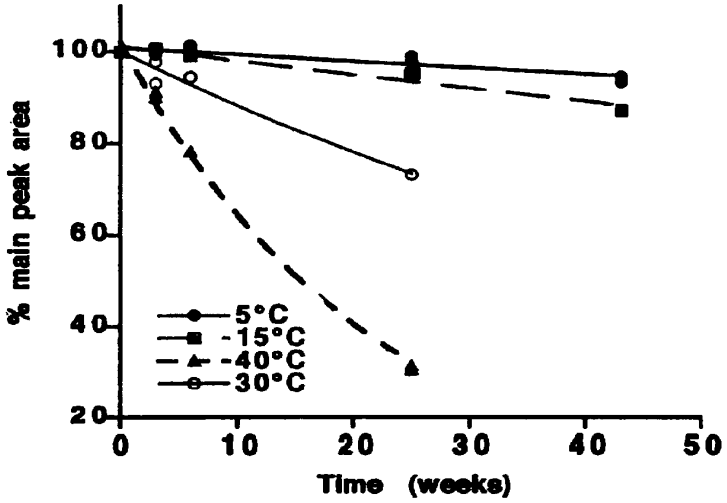


FIG. 19B

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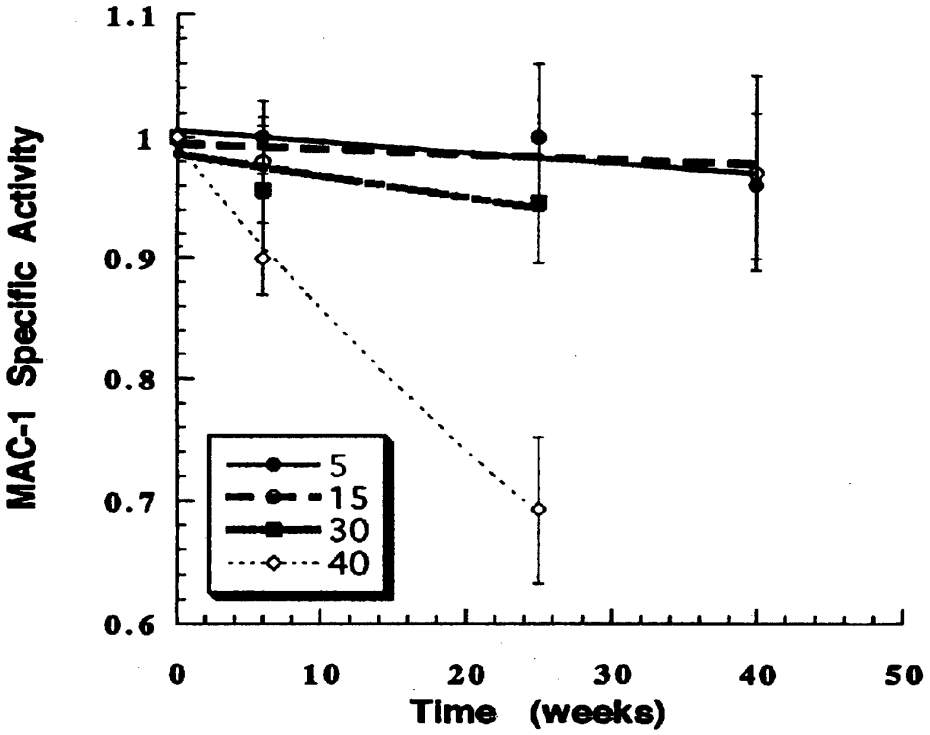


FIG. 19C

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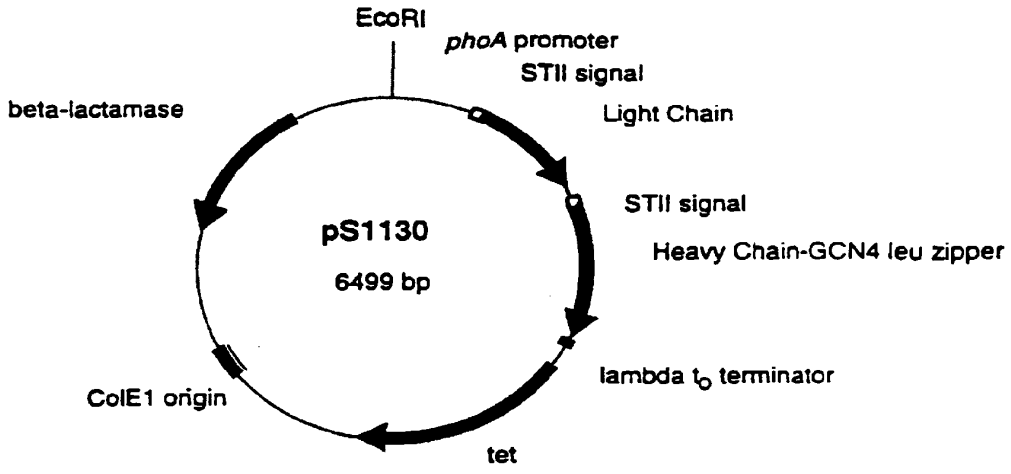


FIG. 20

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```
   1 GAATTCAACT TCTCCATACT TTGGATAAGG AAATACAGAC ATGAAAAATC TCATTGCTGA

  61 GTTGTTATTT AAGCTTTGGA GATTATCGTC ACTGCAATGC TTCGCAATAT GGCGCAAAAT

 121 GACCAACAGC GGTTGATTGA TCAGGTAGAG GGGGCGCTGT ACGAGGTAAA GCCCGATGCC

 181 AGCATTCCTG ACGACGATAC GGAGCTGCTG CGCGATTACG TAAAGAAGTT ATTGAAGCAT

 241 CCTCGTCAGT AAAAAGTTAA TCTTTTCAAC AGCTGTCATA AAGTTGTCAC GGCCGAGACT

 301 TATAGTCGCT TTGTTTTTAT TTTTTAATGT ATTTGTAACT AGAATTCGAG CTCGCCGGGG

 361 ATCCTCTAGA GGTTGAGGTG ATTTT ATG AAA AAG AAT ATC GCA TTT CTT CTT
 -23                           M   K   K   N   I   A   F   L   L

 413 GCA TCT ATG TTC GTT TTT TCT ATT GCT ACA AAC GCG TAC GCT GAT ATC
 -14 A   S   M   F   V   F   S   I   A   T   N   A   Y   A   D   I

 461 CAG ATG ACC CAG TCC CCG AGC TCC CTG TCC GCC TCT GTG GGC GAT AGG
   3 Q   M   T   Q   S   P   S   S   L   S   A   S   V   G   D   R

 509 GTC ACC ATC ACC TGT CGT GCC AGT CAG GAC ATC AAC AAT TAT CTG AAC
  19 V   T   I   T   C   R   A   S   Q   D   I   N   N   Y   L   N

 557 TGG TAT CAA CAG AAA CCA GGA AAA GCT CCG AAA CTA CTG ATT TAC TAT
  35 W   Y   Q   Q   K   P   G   K   A   P   K   L   L   I   Y   Y

 605 ACC TCC ACC CTC CAC TCT GGA GTC CCT TCT CGC TTC TCT GGT TCT GGT
  51 T   S   T   L   H   S   G   V   P   S   R   F   S   G   S   G

 653 TCT GGG ACG GAT TAC ACT CTG ACC ATC AGC AGT CTG CAA CCG GAG GAC
  67 S   G   T   D   Y   T   L   T   I   S   S   L   Q   P   E   D

 701 TTC GCA ACT TAT TAC TGT CAG CAA GGT AAT ACT CTG CCG CCG ACG TTC
  83 F   A   T   Y   Y   C   Q   Q   G   N   T   L   P   P   T   F

 749 GGA CAG GGC ACG AAG GTG GAG ATC AAA CGA ACT GTG GCT GCA CCA TCT
  99 G   Q   G   T   K   V   E   I   K   R   T   V   A   A   P   S

 797 GTC TTC ATC TTC CCG CCA TCT GAT GAG CAG TTG AAA TCT GGA ACT GCC
 115 V   F   I   F   P   P   S   D   E   Q   L   K   S   G   T   A

 845 TCT GTT GTG TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG GCC AAA GTA
 131 S   V   V   C   L   L   N   N   F   Y   P   R   E   A   K   V

 893 CAG TGG AAG GTG GAT AAC GCC CTC CAA TCG GGT AAC TCC CAG GAG AGT
 147 Q   W   K   V   D   N   A   L   Q   S   G   N   S   Q   E   S

 941 GTC ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC AGC CTC AGC AGC ACC
 163 V   T   E   Q   D   S   K   D   S   T   Y   S   L   S   S   T

 989 CTG ACG CTG AGC AAA GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC TGC
 179 L   T   L   S   K   A   D   Y   E   K   H   K   V   Y   A   C

1037 GAA GTC ACC CAT CAG GGC CTG AGC TCG CCC GTC ACA AAG AGC TTC AAC
 195 E   V   T   H   Q   G   L   S   S   P   V   T   K   S   F   N

1085 AGG GGA GAG TGT TAA  G CTGATCCTCT ACGCCGGACG CATCGTGGCG
 211 R   G   E   C
```

FIG. 21A

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```
1131 CTAGTACGCA AGTTCACGTA AAAACGGTAT CTAGAGGTTG AGGTGATTTT  ATG AAA
 -23                                                        M   K

1187 AAG AAT ATC GCA TTT CTT CTT GCA TCT ATG TTC GTT TTT TCT ATT GCT
 -21 K   N   I   A   F   L   L   A   S   M   F   V   F   S   I   A

1235 ACA AAC GCG TAC GCT GAG GTT CAG CTG GTG GAG TCT GGC GGT GGC CTG
  -5 T   N   A   Y   A   E   V   Q   L   V   E   S   G   G   G   L

1283 GTG CAG CCA GGG GGC TCA CTC CGT TTG TCC TGT GCA ACT TCT GGC TAC
  12 V   Q   P   G   G   S   L   R   L   S   C   A   T   S   G   Y

1331 ACC TTT ACC GAA TAC ACT ATG CAC TGG ATG CGT CAG GCC CCG GGT AAG
  28 T   F   T   E   Y   T   M   H   W   M   R   Q   A   P   G   K

1379 GGC CTG GAA TGG GTT GCA GGG ATT AAT CCT AAA AAC GGT GGT ACC AGC
  44 G   L   E   W   V   A   G   I   N   P   K   N   G   G   T   S

1427 CAC AAC CAG AGG TTC ATG GAC CGT TTC ACT ATA AGC GTA GAT AAA TCC
  60 H   N   Q   R   F   M   D   R   F   T   I   S   V   D   K   S

1475 ACC AGT ACA GCC TAC ATG CAA ATG AAC AGC CTG CGT GCT GAG GAC ACT
  76 T   S   T   A   Y   M   Q   M   N   S   L   R   A   E   D   T

1523 GCC GTC TAT TAT TGT GCT AGA TGG CGA GGC CTG AAC TAC GGC TTT GAC
  92 A   V   Y   Y   C   A   R   W   R   G   L   N   Y   G   F   D

1571 GTC CGT TAT TTT GAC GTC TGG GGT CAA GGA ACC CTG GTC ACC GTC TCC
 108 V   R   Y   F   D   V   W   G   Q   G   T   L   V   T   V   S

1619 TCG GCC TCC ACC AAG GGC CCA TCG GTC TTC CCC CTG GCA CCC TCC TCC
 124 S   A   S   T   K   G   P   S   V   F   P   L   A   P   S   S

1667 AAG AGC ACC TCT GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC AAG GAC
 140 K   S   T   S   G   G   T   A   A   L   G   C   L   V   K   D

1715 TAC TTC CCC GAA CCG GTG ACG GTG TCG TGG AAC TCA GGC GCC CTG ACC
 156 Y   F   P   E   P   V   T   V   S   W   N   S   G   A   L   T

1763 AGC GGC GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC TCA GGA CTC TAC
 172 S   G   V   H   T   F   P   A   V   L   Q   S   S   G   L   Y

1811 TCC CTC AGC AGC GTG GTG ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG
 188 S   L   S   S   V   V   T   V   P   S   S   S   L   G   T   Q

1859 ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC AGC AAC ACC AAG GTC GAC
 204 T   Y   I   C   N   V   N   H   K   P   S   N   T   K   V   D

1907 AAG AAA GTT GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA TGC CCG CCG
 220 K   K   V   E   P   K   S   C   D   K   T   H   T   C   P   P

1955 TGC CCA GCA CCA GAA CTG CTG GGC GGC CGC ATG AAA CAG CTA GAG GAC
 236 C   P   A   P   E   L   L   G   G   R   M   K   Q   L   E   D

2003 AAG GTC GAA GAG CTA CTC TCC AAG AAC TAC CAC CTA GAG AAT GAA GTG
 252 K   V   E   E   L   L   S   K   N   Y   H   L   E   N   E   V

2051 GCA AGA CTC AAA AAG CTT GTC GGG GAG CGC TAA  GCATGCG ACGGCCCTAG
 268 A   R   L   K   K   L   V   G   E   R

2101 AGTCCCTAAC GCTCGGTTGC CGCCGGGCGT TTTTTATTGT TAA
```

FIG. 21B

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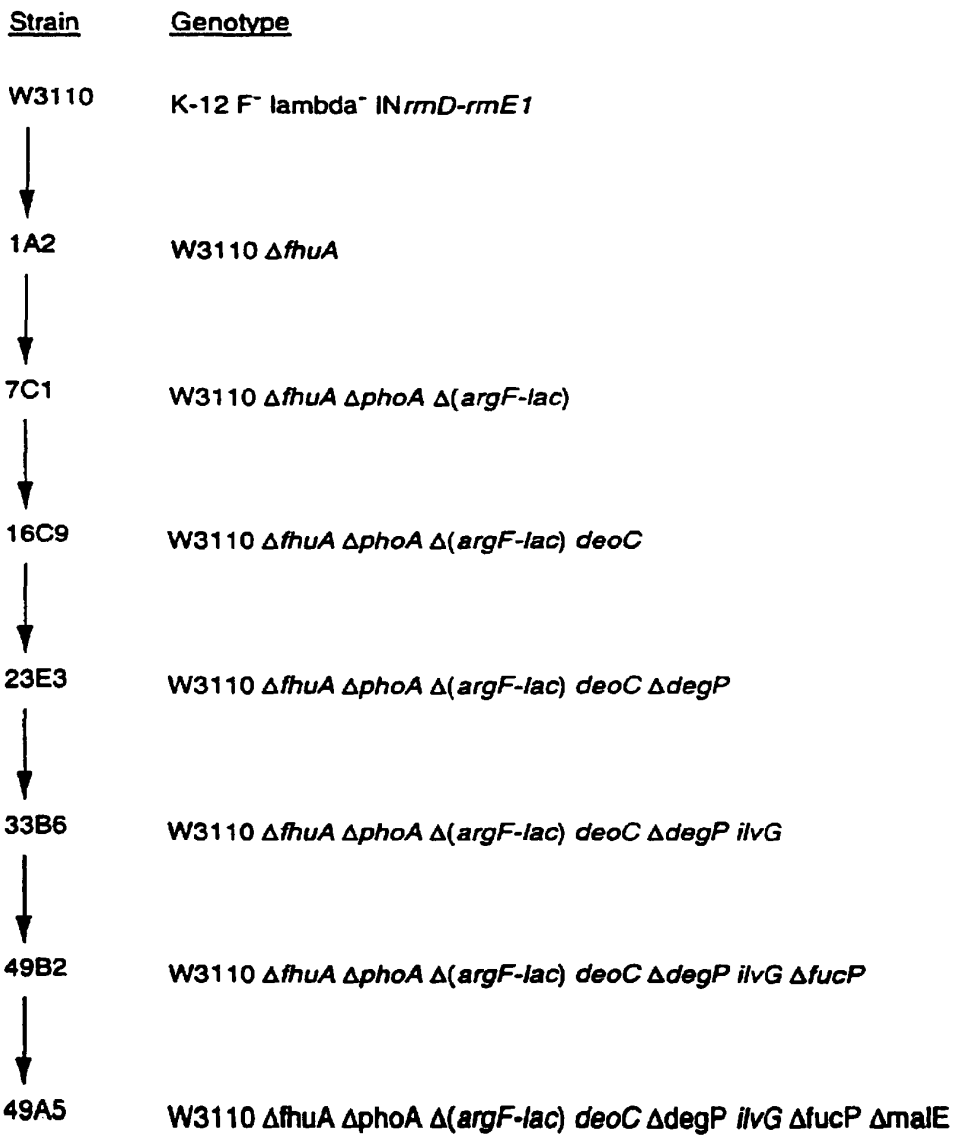


FIG. 22

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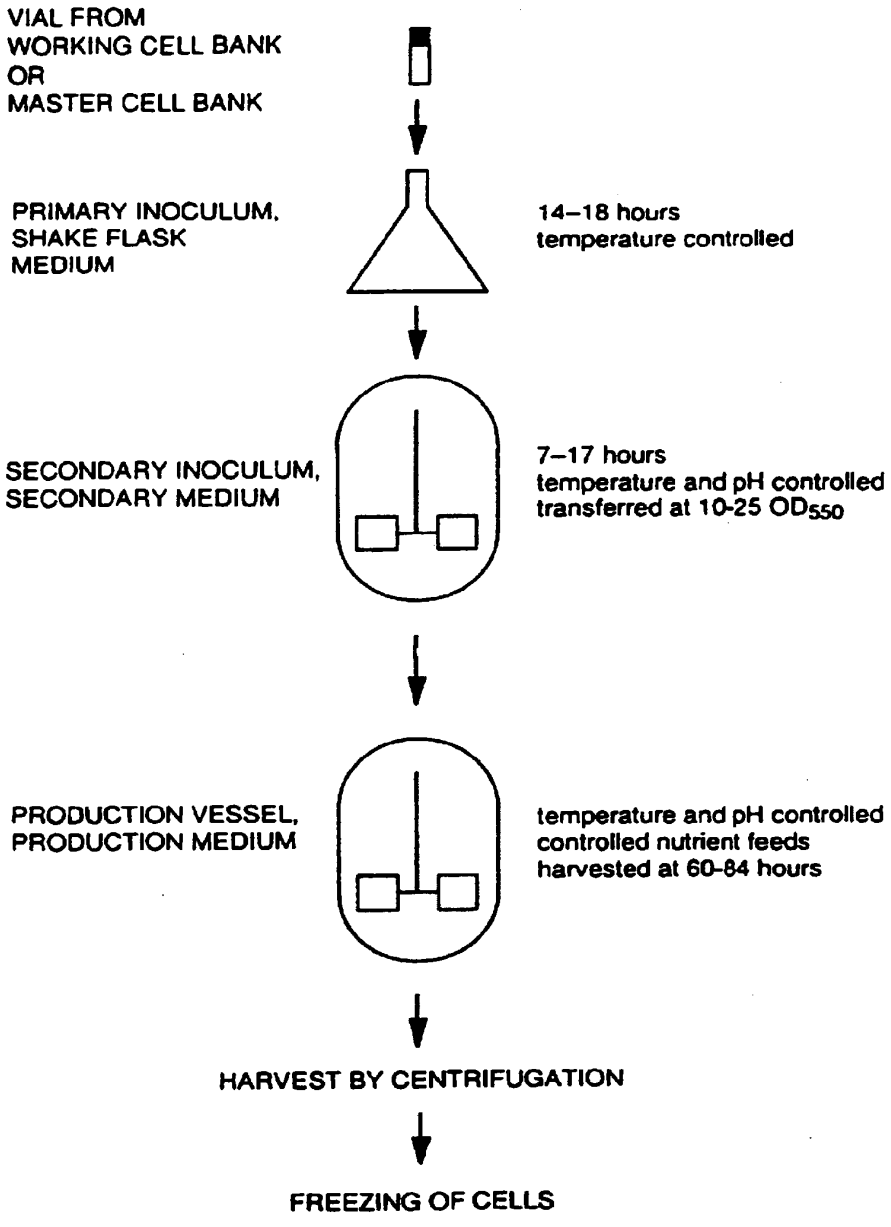


FIG. 23

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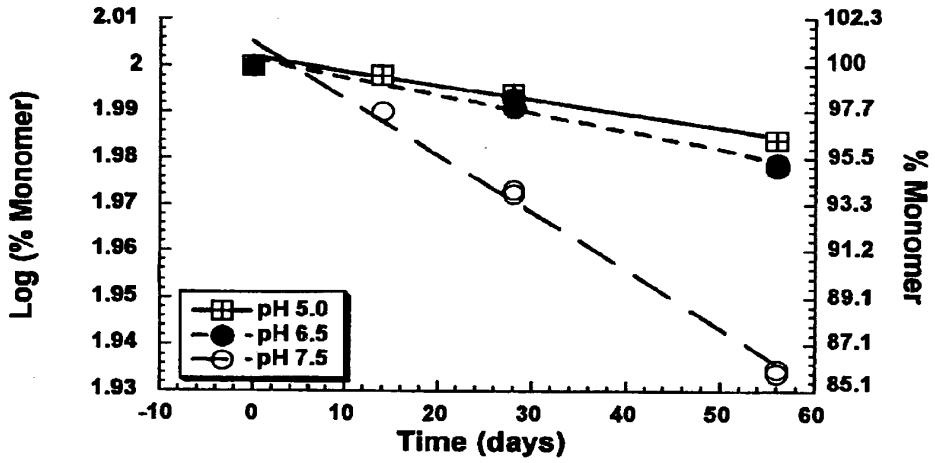


FIG. 24A

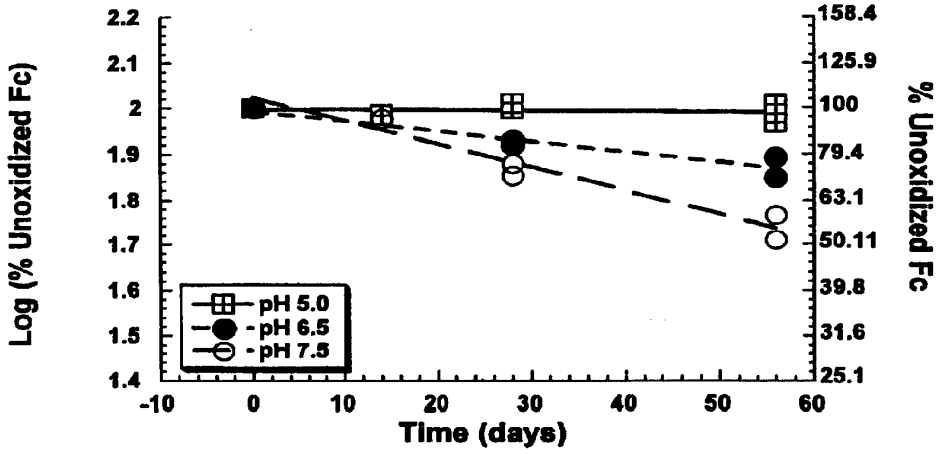


FIG. 24B

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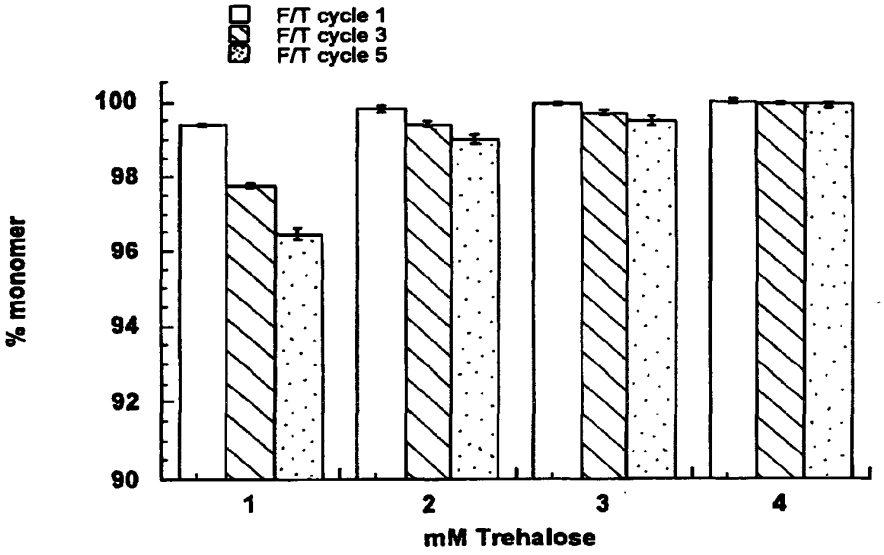


FIG. 25

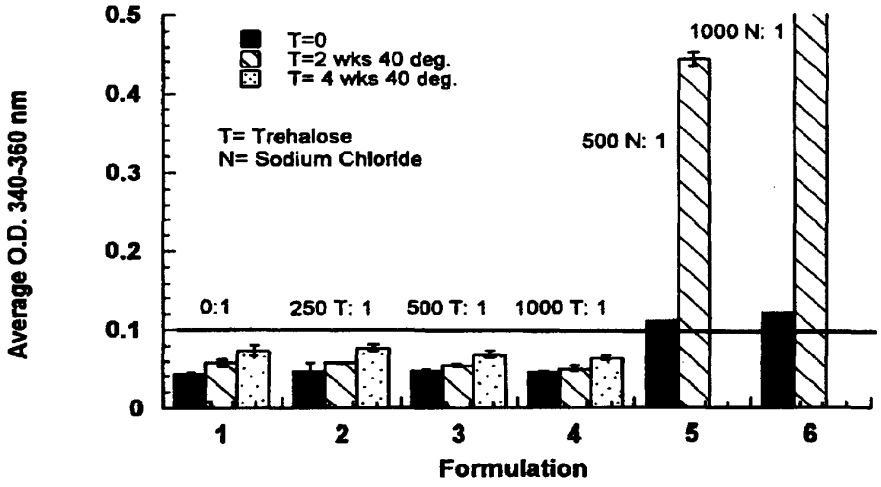


FIG. 26

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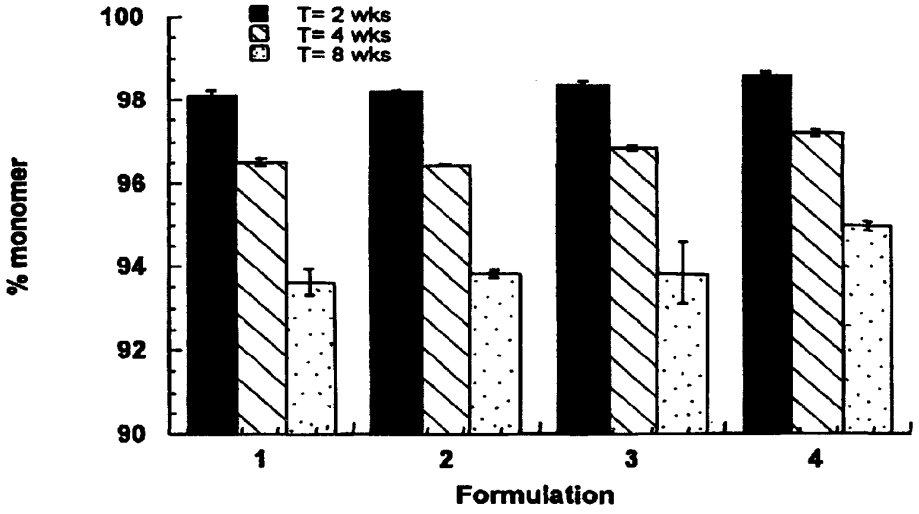


FIG. 27

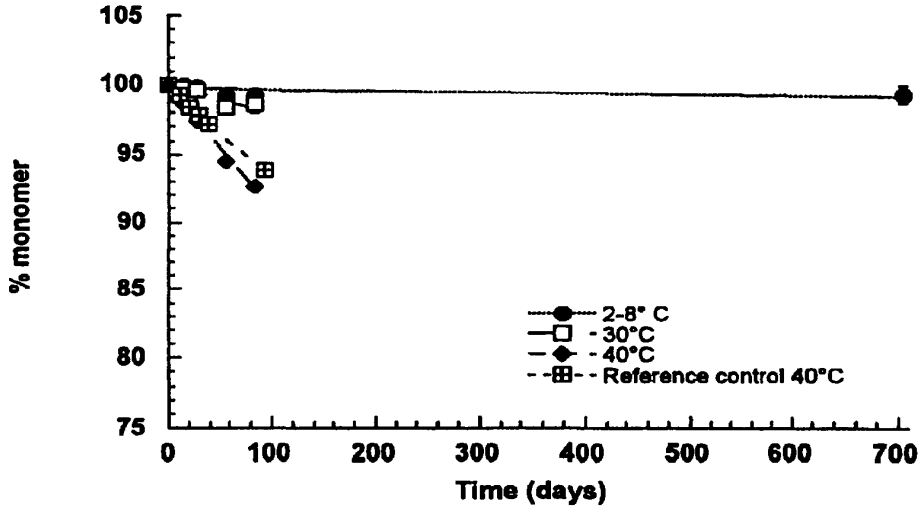


FIG. 28

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ANTIBODY FORMULATION

RELATED APPLICATION

This is a non-provisional application claiming priority under Section 119(e) to provisional application 60/053,087, filed on Jun. 13, 1997, the contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

This invention is directed to a stable aqueous pharmaceutical formulation comprising an antibody.

BACKGROUND OF THE INVENTION

In the past ten years, advances in biotechnology have made it possible to produce a variety of proteins for pharmaceutical applications using recombinant DNA techniques. Because proteins are larger and more complex than traditional organic and inorganic drugs (i.e. possessing multiple functional groups in addition to complex three-dimensional structures), the formulation of such proteins poses special problems. For a protein to remain biologically active, a formulation must preserve intact the conformational integrity of at least a core sequence of the protein's amino acids while at the same time protecting the protein's multiple functional groups from degradation. Degradation pathways for proteins can involve chemical instability (i.e. any process which involves modification of the protein by bond formation or cleavage resulting in a new chemical entity) or physical instability (i.e. changes in the higher order structure of the protein). Chemical instability can result from deamidation, racemization, hydrolysis, oxidation, beta elimination or disulfide exchange. Physical instability can result from denaturation, aggregation, precipitation or adsorption, for example. The three most common protein degradation pathways are protein aggregation, deamidation and oxidation. Cleland et al *Critical Reviews in Therapeutic Drug Carrier Systems* 10(4): 307–377 (1993).

Included in the proteins used for pharmaceutical applications are antibodies. An example of an antibody useful for therapy is an antibody which binds to the CD18 antigen. CD18 is the common β subunit of three heterodimeric membrane integrins restricted to leukocytes that mediate trafficking and adhesion to the vascular endothelium, particularly at sites of inflammation (for reviews see Hynes, R. O. *Cell*, 69:11–25 (1992); Stoolman, *Cell*, 56:907–910 (1989); Jutila et al. *Transplantation* 48(5): 727–731 (1989); Springer, T. A., *Nature* 346:425–434 (1990); and Albelda and Buck, *FASEB J.* 4:2868–2880 (1990)). The heterodimer containing CD18 and CD11b (also called MAC-1) is found primarily on neutrophils, monocytes, and some lymphocytes whose normal interaction with ICAM-1 on vascular endothelium mediates adhesion and "rolling" of cells along the vasculature. In severe hemorrhagic trauma with concurrent decrease in cardiac output and ischemia, early (within 30 min) neutrophil activation (in response to released cytokines) and up-regulation of MAC-1 increases neutrophil "stickiness". This precedes extravasation and release of proteases and superoxides that ultimately lead to further tissue damage and increased vascular permeability (Hernandez et al, *Am. J. Physiol.*, 253(3 Pt 2): H699-H703 (1987)). Reperfusion following resuscitation exacerbates the edema and necrosis, and leads to multi-organ failure and death. Early treatment with monoclonal antibodies to CD18 in a partally-severed, ischemic rabbit ear trauma model alleviated tissue necrosis following reattachment (Vedder et al., *J. Clin. Invest.* 81:939–944 (1988)). A humanized antibody showed efficacy in reducing multi-organ damage and death in a rhesus monkey model of decreased cardiac output (created by depletion of ⅔ of blood volume for ~2 hours (Mileski etal., *Surgery*, 108(2):206–212 (1990)). These studies point to the therapeutic potential of anti-CD18 antibodies for acute treatment of hemorrhagic shock.

Another antigen of interest for targeting with antibodies is the CD20 antigen, also known as "Bp35". CD20 is a human B cell marker which is expressed during early pre-B cell development and remains until plasma cell differentiation. The CD20 molecule may regulate a step in the activation process which is required for cell cycle initiation and differentiation and is usually expressed at very high levels on neoplastic B cells. Thus, the CD20 surface antigen can be targeted for treating B cell lymphomas. U.S. Pat. No. 5,736,137 issued Apr. 7, 1998 describes the chimeric antibody "C2B8" which binds the CD20 antigen and its use to treat B cell lymphoma.

There is a need in the art for a stable aqueous pharmaceutical formulation comprising an antibody, such as an anti-CD18 or anti-CD20 antibody, which is suitable for therapeutic use.

SUMMARY OF THE INVENTION

Accordingly, the invention provides a stable aqueous pharmaceutical formulation comprising a therapeutically effective amount of an antibody not subjected to prior lyophilizabon, a buffer maintaining the pH in the range from about 4.5 to about 6.0, a surfactant and a polyol. Preferably the formulation is stable at a temperature of about 2–8° C. for at least one year, and/or is stable at a temperature of about 30° C. for at least one month and/or is stable following freezing and thawing of the formulation.

The invention also relates to an article of manufacture comprising a container holding a stable aqueous pharmaceutical formulation comprising a therapeutically effective amount of an antibody not subjected to prior lyophilizabon, a buffer maintaining the pH in the range from about 4.5 to about 6.0, a surfactant and a polyol.

In yet a further aspect, the invention relates to a method for stabilizing an antibody in an aqueous pharmaceutical formulation by combining a therapeutically effective amount of an antibody not subjected to prior lyophilization, a buffer maintaining the pH in the range from about 4.5 to about 6.0, a surfactant and a polyol.

In a still further aspect, the invention concerns a method of treating a mammal comprising administering a therapeutically effective amount of the aqueous pharmaceutical formulation disclosed herein to a mammal, wherein the mammal has a disorder requiring treatment with the antibody in the formulation. Where the antibody binds CD18, examples of disorders to be treated include hemorrhagic shock, thermal injury (such as that resulting from burns), stroke (including ischemic and hemorrhagic stroke) and myocardial infarction. For an anti-IL8 antibody, disorders to be treated include inflammatory disorders such as adult respiratory distress syndrome (ARDS), hypovolemic shock, ulcerative colitis, and rheumatoid arthritis. Where the antibody binds CD20, disorders to be treated include B cell lymphomas.

These and further aspects of the invention will be apparent to those skilled in the art.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A and 1B depict the amino acid sequence of rhuMAb CD18 heavy chain (FIG. 1A; SEQ ID NO:1) and

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light chain (FIG. 1B; SEQ ID NO:2). The sequence in italics in FIG. 1A (SEQ ID NO:3) is that of the leucine zipper.

FIGS. 2A and 2B are hydroflex plots of rhuMAb CD18 heavy chain (FIG. 2A) and light chain (FIG. 2B). Kyte-Dooliutle hydrophobicity calculations averaged with a window of 6 amino acids were made on the protein sequence. Flexibility values were estimated from the product of the hydrophobicity of each residue and its side chain volume (again averaged over a window of 6 residues). Asn-Gly and Asn-Ser motifs in flexible regions are more likely to deamidatc than those in morc rigid structurcs. Thc CDRs arc also shown. Most of the heavy chain methionines and heavy chain Asn 84 are near the CDR's.

FIGS. 3A and 3B show the effect of storage temperature on light scattering in different rhuMAb CD18 formulations after 5 weeks storage at the designated temperatures as measured by average absorbance in the range of 340–360 nm (FIG. 3A), or the ratio of A278 over A252 nm (FIG. 3B). RhuMAb CD18 in formulation F3 is prone to the formation of insoluble aggregates.

FIG. 4 shows the effect of storage temperature on protein concentration of rhuMAb CD18 formulations measured by absorbance at 278 nm corrected for vehicle absorbance at 320 nm.

FIGS. 5A and 5B depict the effect of storage temperature on the stability of rhuMAb CD18 formulation F2 (FIG. 5A) and formulation F5 (FIG. 5B) assayed by size exclusion chromatography (SEC). A smaller MW species (see arrows) appeared which was more pronounced at pH 6 (FIG. 5B) compared to pH 5 (FIG. 5A).

FIGS. 6A and 6B represent the effect of storage time and temperature on the stability of rhuMAb CD18 showing total peak area (FIG. 6A), and % main peak (FIG. 6B) for all formulations, assayed by SEC. No significant change in total peak area was noted. F1 and F5 maintained the lowest % main peak.

FIG. 7 is an Arrhenius plot of the % main peak area from SEC of rhuMAb CD18 formulated in acetate with trehalose at pH 5 (F2). Activation Energy=19±6 kcal/mole.

FIG. 8 shows the effect of storage for 5 weeks at 40° C. on different rhuMAb CD18 formulations, assayed by hydrophobic interaction chromatography (HIC). The early eluting peaks at 17.5 min and the shoulder at the leading edge of the main peak increased compared to controls at −70° C. F2 showed the least increase in both components (see FIG. 9).

FIG. 9 shows the effect of storage for 5 weeks at different temperatures on formulation F2, assayed by HIC. A pre-main peak became apparent compared to −70° C. control.

FIG. 10 depicts the effect of storage temperature on the stability of rhuMAb CD18 (5 week data) assayed by HIC showing total peak area recovered. A trend towards loss in area was noted in all formulations with increasing storage temperatures, except for F5 which started lower to begin with.

FIG. 11 shows the effect of storage for 5 weeks at different temperatures on formulation F2, assayed by reverse phase-hydrophobic liquid chromatography (RP-HPLC). A small partially resolved pre-main peak component (see arrow) increased at higher temperatures, while its slightly earlier eluting neighbor remained unchanged.

FIG. 12 shows the effect of storage for 5 weeks on the stability of rhuMAb CD18 assayed by RP-HPLC showing percentage of the main peak. F2 and F3 showed the highest % main peak after 5 weeks at 40° C.

FIG. 13 is an Arrhenius plot based on the RP-HPLC % main peak for formulation F2. Activation energy (~20 kcal/mole) was only approximated from these data due to the very small K for 5° C., it appears similar to that obtained with the SEC and ion-exchange HPLC (IEX) assays.

FIGS. 14A and 14B show the effect of storage temperature on the stability of rhuMAb CD18, assayed by IEX, on formulation F2 at pH 5 (FIG. 14A) and F5 at pH 6 (FIG. 14B). Two pre-main peaks increased with increasing time and temperature, more pronounced at pH 6. A hump from 22 to 28 minutes is an artifact due to an impurity washing off the column.

FIG. 15 is an Arrhenius plot of the % main peak area from IEX of rhuMAb CDI8 formulated in acetate with trehalose at pH 5 (F2). Activation Energy=20±10 kcal/mole.

FIG. 16 shows the three dimensional structure of rhuMAb CD18, including positions of methionine residues. Met 65 and Met 83 are exposed, whereas others are buried in the structure and are expected to be less labile to oxidation.

FIG. 17 shows fluorescence spectroscopy of rhuMAb CD18 formulations. The emission spectra of various formulations were obtained on an SLM8000 fluorimeter, using 280 nm excitation wavelength. 500 μL samples were placed in quartz cuvcftes and spectra wcrc obtained with a 2nm bandwidth at 22° C. Samples were prepared at the different pHs by dilution of a concentrated stock to 0.1 mg/mL protein and analyses were made after 24 hr at 25° C. The antibody is conformationally stable above pH 3.

FIGS. 18A and 18B depict the effect of pH and protein concentration on the stability of rhuMAb CD18. Formulations in the pH range of 3 to 6 and concentration range of 0.5 to 25 mg/mL were placed on stability at 40° C. and −70° C. for 2 months. Analyses were made by IEX (FIG. 18A) and SEC (FIG. 18B). pH 5 was found to be the preferred pH, irrespective of protein concentration.

FIGS. 19A, 19B and 19C depict the kinetics of degradation of rhuMAb CD18 by SEC (FIG. 19A), IEX (FIG. 19B) and MAC-1 binding (FIG. 19C) at various temperatures. Duplicate samples in 10 mM Na acetate, 8% trehalose, 0.01% TWEEN 20™, pH 5 were prepared in 3 cc glass vials and placed on stability at the indicated temperatures. In FIG. 19C, specific activity was the ratio of MAC-1 binding to total $F(ab')_2$ ELISA. Samples are stable at 5° C. and 15° C. up to 43 weeks and at 30° C. up to 1 month. Analyses were made at the time points indicated.

FIG. 20 depicts the structure of plasmid pS1130 used to produce rhuMAb CD18 of the example below.

FIGS. 21A and 21B depict the full nucleotide sequence (SEQ ID NO:9) and encoded amino acid sequences (SEQ ID No's:10 and 11, respectively) of the pS 1130 expression cassette.

FIG. 22 shows derivation of the 49A5 production cell line.

FIG. 23 is a schematic of the fermentation process for rhuMAb CD18.

FIGS. 24A and 24B depict the effect of pH on the rate of aggregation (FIG. 24A) and oxidation (FIG. 24B) of 40 mg/mL rhuMAb CD20, 25 mM histidine, 0.02% polysorbate formulations at pH 5,6.5 or 7.5 stored at 40° C.

FIG. 25 depicts the effect of excess molar ratios of trehalose on the freeze-thaw induced aggregation of rhuMAb CD20 multidose formulations. Each formulation is composed of 40 mg/mL rhuMAb CD20, 20 mM acetate, 0 to 1000:1 molar ratio of trehalose, 0.9% benzyl alcohol and 0.02% polysorbate 20, pH 5.0 The amount of trehalose in formulations 1–4 was follows: 1=0 moles trehalose: 1 mole of rhuMAb CD20 (0 mM trehalose); 2=250 moles trehalose:

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1 mole rhuMAb CD20 (67 mM trehalose); 3=500 moles trehalose: 1 mole rhuMAb CD20 (134 mM trehalose); and 4=1000 moles trehalose: 1 mole rhuMAb CD20 (267 mM trehalose).

FIG. **26** shows the effect of excess molar ratios of trehalose or sodium chloride on the clarity of rhuMAb CD20 multdose formulations stored at 40° C. for up to four weeks. The composition of the formulations is 40 mg/mL rhuMAb CD20,20 mM acetate, 0 to 1000:1 molar ratio of trehalose or sodium chloride, 0.9% benzyl alcohol and 0.02% polysorbate 20 at pH 5.0. The clarity of the 500:1 and 1000:1 molar ratio of sodium chloride to rhuMAb CD20 formulations was not measured after four weeks at 40° C. due to the physical appearance of these formulations. The 500:1 ratio formulation was very opalescent while the 1000:1 ratio formulabon had separated into two phases composed of a thin opaque gel layer covered with an opalescent fluid. The O.D. of the 1000:1 molar ratio of sodium chloride to rhuMAb CD20 formulation was 2.72 after two weeks storage at 40° C.

FIG. **27** depicts the effect of excess molar ratios of trehalose on the stability of rhuMAb CD20 muffidose formulations stored at 40° C. as analyzed by SEC HPLC. The composition of the formulations is 40 mg/mL rhuMAb CD20, 20 mM acetate, 0–267 mM trehalose, 0.9% benzyl alcohol and 0.02% polysorbate 20 at pH 5.0. The amount of trehalose in formulations 1–4 was as follows: 1=0 moles trehalose: 1 mole of rhuMAb CD20 (0 mM trehalose); 2=250 moles trehalose: 1 mole rhuMAb CD20 (67 mM trehalose); 3=500 moles trehalose: 1 mole rhuMAb CD20 (134 mM trehalose); and 4=1000 moles trehalose: 1 mole rhuMAb CD20 (267 mM trehalose). The percent monomer at each bmepoint was normalized to the percent monomer at T=0.

FIG. **28** shows the stability profile of the prototype liquid rhuMAb CD20 multidose formulation stored at 2–8° C. for up to two years as measured by SEC HPLC. The formulation was composed of 40 mg/mL rhuMAb CD20, 150 mM trehalose, 0.9% benzyl alcohol and 0.02% polysorbate 20 at pH 5.0. The percent monomer at each timepoint was normalized to the percent monomer at T=0. The bioactivity of the formulation stored at 2–8° C. for two years was 99.2% relative to the reference control as measured by the CDC assay.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

The term "pharmaceutical formulation" refers to preparations which are in such form as to permit the biological actvity of the active ingredients to be unequivocally effective, and which contain no additional components which are toxic to the subjects to which the formulation would be administered. "Pharmaceutically acceptable" excipients (vehicles, additives) are those which can reasonably be administered to a subject mammal to provide an effective dose of the active ingredient employed.

A "stable" formulation is one in which the protein therein essentially retains its physical stability and/or chemical stability and/or biological activity upon storage. Various analytical techniques for measuring protein stability are available in the art and are reviewed in *Peptide and Protein Drug Delivery*, 247–301, Vincent Lee Ed., Marcel Dekker, Inc., New York, N.Y., Pubs. (1991) and Jones, A. *Adv. Drug Delivery Rev.* 10: 29–90 (1993), for example. Stability can be measured at a selected temperature for a selected time period. Preferably, the formulation is stable at room temperature (~30° C.) or at 40° C. for at least 1 month and/or stable at about 2–8° C. for at least 1 year for at least 2 years. Furthermore, the formulation is preferably stable following freezing (to, e.g., −70° C.) and thawing of the formulation.

A protein "retains its physical stability" in a pharmaceutical formulation if it shows no signs of aggregation, precipitation and/or denaturation upon visual examination of color and/or clarity, or as measured by UV light scattering or by size exclusion chromatography.

A protein "retains its chemical stability" in a pharmaceutical formulation, if the chemical stability at a given time is such that the protein is considered to still retain its biological activity as defined below. Chemical stability can be assessed by detecting and quantifying chemically altered forms of the protein. Chemical alteration may involve size modification (e.g. clipping) which can be evaluated using size exclusion chromatography, SDS-PAGE and/or matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI/TOF MS), for example. Other types of chemical alteration include charge alteration (e.g. occurring as a result of deamidation) which can be evaluated by ion-exchange chromatography, for example.

An antibody "retains its biological activity" in a pharmaceutical formulation, if the biological activity of the antibody at a given time is within about 10% (within the errors of the assay) of the biological activity exhibited at the time the pharmaceutical formulation was prepared as determined in an antigen binding assay, for example. Other "biological activity" assays for antibodies are elaborated hereinbelow.

By "isotonic" is meant that the formulation of interest has essentially the same osmotic pressure as human blood. Isotonic formulations will generally have an osmotic pressure from about 250 to 350 mOsm. Isotonicity can be measured using a vapor pressure or ice-freezing type osmometer, for example.

A "polyol" is a substance with multiple hydroxyl groups, and includes sugars (reducing and nonreducing sugars), sugar alcohols and sugar acids. Preferred polyols herein have a molecular weight which is less than about 600 kD (e.g. in the range from about 120 to about 400 kD). A "reducing sugar" is one which contains a hemiacetal group that can reduce metal ions or react covalently with lysine and other amino groups in proteins and a "nonreducing sugar" is one which does not have these properties of a reducing sugar. Examples of reducing sugars are fructose, mannose, maltose, lactose, arabinose, xylose, ribose, rhamnose, galactose and glucose. Nonreducing sugars include sucrose, trehalose, sorbose, melezitose and raffinose. Mannitol, xylitol, erythritol, threitol, sorbitol and glycerol are examples of sugar alcohols. As to sugar acids, these include L-gluconate and metallic salts thereof. Where it desired that the formulation is freeze-thaw stable, the polyol is preferably one which does not crystallize at freezing temperatures (e.g. −20° C.) such that it destabilizes the antibody in the formulation. Nonreducing sugars such as sucrose and trehalose are the preferred polyols herein, with trehalose being preferred over sucrose, because of the superior solution stability of trehalose.

As used herein, "buffer" refers to a buffered solution that resists changes in pH by the action of its acid-base conjugate components. The buffer of this invention has a pH in the range from about 4.5 to about 6.0; preferably from about 4.8 to about 5.5; and most preferably has a pH of about 5.0. Examples of buffers that will control the pH in this range include acetate (e.g. sodium acetate), succinate (such as

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sodium succinate), gluconate, histidine, citrate and other organic acid buffers. Where a freeze-thaw stable formulation is desired, the buffer is preferably not phosphate.

In a pharmacological sense, in the context of the present invention, a "therapeutically effective amount" of an antibody refers to an amount effective in the prevention or treatment of a disorder for the treatment of which the antibody is effective. A "disorder" is any condition that would benefit from treatment with the antibody. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question.

A "preservative" is a compound which can be included in the formulation to essentially reduce bacterial action therein, thus facilitating the production of a multi-use formulation, for example. Examples of potential preservatives include octadecyldimethylbenzyl ammonium chloride, hexamethonium chloride, benzalkonium chloride (a mixture of alkylbenzyldimethylammonium chlorides in which the alkyl groups are long-chain compounds), and benzethonium chloride. Other types of preservatives include aromatic alcohols such as phenol, butyl and benzyl alcohol, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol, 3-pentanol, and m-cresol. The most preferred preservative herein is benzyl alcohol.

As used herein, the term "inflammatory disorders" refers to pathological states resulting in inflammation, e.g. caused by influx of leukocytes and/or neutrophil chemotaxis. Inflammation may result from infection with pathogenic organisms and viruses and noninfectious means such as trauma or reperfusion following myocardial infarction or stroke, immune response to foreign antigen and autoimmune responses. Examples of inflammatory disorders include inflammatory skin diseases such as psoriasis and dermatitis; responses associated with inflammatory bowel disease (such as Crohn's disease and ulcerative colitis); ischemic reperfusion; adult respiratory distress syndrome; meningitis; encephalitis; uvei.s; autoimmune diseases such as rheumatoid arthritis, Sjorgen's syndrome, vasculitis; diseases involving leukocyte diapedesis; central nervous system (CNS) inflammatory disorder; multiple organ injury syndrome secondary to septcaemia or trauma; alcoholic hepatitis; bacterial pneumonia; antigen-antibody complex mediated diseases; hypovolemic shock; glomerulonephritis; multiple sclerosis; Type l diabetes mclitis; acute and delayed hypersensitivity; graft vs. host disease; transplant rejection; reperfusion injury; endotoxic shock; disease states due to leukocyte dyscrasia and metastasis; asthma; pulmonary oxygen toxicity; inflammation of the lung, including pleurisy, alveolitis, vasculitis, pneumonia, chronic bronchitis, bronchiectasis, and cystic fibrosis; etc.

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they exhibit the desired biological activity.

"Antibody fragments" comprise a portion of a full length antibody, generally the antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antgenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is notto be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature* 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al, *Nature* 352:624–628 (1991) and Marks et al., *J. Mol. Biol.* 222:581–597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al., *Proc. NatL. Acad Sci. USA* 81:6851–6855 (1984)).

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (i.e. residues 24–34 (L1), 50–56 (L2) and 89–97 (L3) in the light chain variable domain and 31–35 (H1), 50–65 (H2) and 95–102 (H3) in the heavy chain variable domain; Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)) and/or those residues from a "hypervariable loop"(i.e. residues 26–32 (L1), 50–52 (L2) and 91–96 (L3) in the light chaiin variable domain and 26–32 (H1), 53–55 (H2) and 96–101 (H3) in the heavy chain variable domain; Chothia Lesk *J. Mol. Biol.* 196:901–917 (1987)). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined. The CDR and FR residues of the H52 antibody of the example below are identified in Eigenbrot et al. *Proteins: Structure, Function and Genetics* 18:49–62 (1994).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, FR residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine

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antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature* 321:522–525 (1986); Riechmann et al, *Nature* 332:323–329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593–596 (1992).

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

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

The expression "linear antibodies" when used throughout this application refers to the antibodies described in Zapata et al. *Protein Eng.* 8(10):1057–1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H–C_H1–V_H–C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

The antibody which is formulated is preferably essentially pure and desirably essentially homogeneous (i.e. free from contaminating proteins etc). "Essentially pure" antibody means a composition comprising at least about 90% by weight of the antibody, based on total weight of the composition, preferably at least about 95% by weight. "Essentially homogeneous" antibody means a composition comprising at least about 99% by weight of antibody, based on total weight of the composition.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

II. Modes for Carrying Out the Invention

The invention herein relates to a stable aqueous formulation comprising an antibody. The antibody in the formulation is prepared using techniques available in the art for generating antibodies, exemplary methods of which are described in more detail in the following sections.

The antibody is directed against an antigen of interest. Preferably, the antigen is a biologically important polypeptde and administration of the antibody to a mammal suffering from a disorder can result in a therapeutic benefit in that mammal. However, antibodies directed against non-polypeptide antigens (such as tumor-associated glycolipid antigens; see U.S. Pat. No. 5,091,178) are also contemplated.

Where the antigen is a polypeptide, it may be a transmembrane molecule (e.g. receptor) or ligand such as a growth factor. Exemplary antigens include molecules such as renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tssue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tssue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1–3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19 and CD20; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrns such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments of any of the above-listed polypeptides.

Preferred molecular targets for antibodies encompassed by the present invention include CD proteins such as CD3, CD4, CD8, CD19, CD20 and CD34; members of the ErbB receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mac1, p150,95, VLA-4, ICAM-1, VCAM and αv/β3 integrin including either α or β subunits thereof (e.g. anti-CD11a, anti-CD18or anti-CD11b antibodies); growth factors such as VEGF; an interleukin such as IL8; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; mpl receptor; CTLA-4; protein C etc.

The preferred antibody herein is one which binds to human CD18 and preferably blocks (partially or completely) the ability of a cell (e.g. a neutrophil) expressing the CD18 subunit at its cell surface to bind to endothelium. Examples

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of anti-CD18 antibodies include MHM23 (Hildreth et al., *Eur. J. Immunol.* 13:202–208 (1983)); M18/2 (IgG_{2a}; Sanches-Madrd et al, *J. Exp. Med.*158:586 (1983)); H52 (American Type Culture Collection (ATCC) Deposit HB 10160); Mas191c and IOT18 (Vermot Desroches et al., *Scand. J. Immunol.* 33:277–286 (1991)); and NA-8 (WO 94/12214). The preferred antibody is one which binds to the CD18 epitope to which either MHM23 or H52 binds. In certain embodiments, the antibody may bind to a region in the extracellular domain of CD18 which associates with CD11b and the antibody may also dissociate α and β chains (e.g. the antibody may dissociate the CD11b and CD18 complex as is the case for the MHM23 antibody).

Techniques for producing antibodies which can be formulated as disclosed herein will be elaborated below.

A. Antibody Preparation

(i) Antigen Preparation

Soluble antigens or fragments thereof, optionally conjugated to other molecules, can be used as immunogens for generating antibodies. For transmembrane molecules, such as receptors, fragments of these (e.g. the extracellular domain of a receptor) can be used as the immunogen. Alternatively, cells expressing the transmembrane molecule can be used as the immunogen. Such cells can be derived from a natural source (e.g. cancer cell lines) or may be cells which have been transformed by recombinant techniques to express the transmembrane molecule. Other antigens and forms thereof useful for preparing antibodies will be apparent to those in the art.

(ii) Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with ⅕ to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(iii) Monoclonal Antibodies

Monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster or macaque monkey, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59–103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur et al, *Monoclonal Antibody Production Techniques and Applications*, pp. 51–63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59–103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Recombinant production of antibodies will be described in more detail below.

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries gener-

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ated using the techniques described in McCafferty et al., *Nature*, 348:552–554 (1990).

Clackson et al., *Nature*, 352:624–628 (1991) and Marks etal., *J. Mol. Biol.*, 222:581–597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology*, 10:779–783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse etal., *Nuc. Acids. Res.*, 21:2265–2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, et al., *Proc. Natl Acad. Sci. USA*, 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iv) Humanized and Human Antibodies

A humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature*, 321:522–525 (1986); Riechmann et al., *Nature*, 332:323–327 (1988); Verhoeyen et al., *Science*, 239:1534–1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims et al., *J. Immunol.*, 151:2296 (1993); Chothia et al., *J. Mol. Biol.*, 196:901 (1987)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad Sci. USA*, 89:4285 (1992); Presta et al., *J. Immnol.*, 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al, *Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits et al., *Nature*, 362:255–258 (1993); Bruggermann et al., *Year in Immuno.*, 7:33 (1993); and Duchosal et al. *Nature* 355:258 (1992). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al, *J. Mol. Biol.*, 227:381 (1991); Marks et al, *J. MoL Biol.*, 222:581–597 (1991); Vaughan et al. *Nature Biotech* 14:309 (1996)).

(v) Antibody Fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107–117 (1992) and Brennan et al., *Science*, 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., Bio/Technology 10:163–167 (1992)). In another embodiment as described in the example below, the $F(ab')_2$ is formed using the leucine zipper GCN4 to promote assembly of the $F(ab')_2$ molecule. According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185.

(vi) Multispecific Antibodies

Multspecific antibodies have binding specificities for at least two different epitopes, where the epitopes are usually from different antigens. While such molecules normally will only bind two different epitopes (i.e. bispecific antibodies, BsAbs), antibodies with additional specificities such as trispecific antibodies are encompassed by this expression when used herein. Examples of BsAbs include those with one arm directed against a tumor cell antigen and the other

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arm directed against a cytotoxic trigger molecule such as anti-FcγRI/anti-CD15, anti-p185^{HER2}/FcγRIII (CD16), anti-CD3/anti-malignant B-cell (1D10), anti-CD3/anti-p185^{HER2}, anti-CD3/anti-p97, anti-CD3/anti-renal cell carcinoma, anti-CD3/anti-OVCAR-3, anti-CD3/L-D1 (anti-colon carcinoma), anti-CD3/anti-melanocyte stimulating hormone analog, anti-EGF receptor/anti-CD3, anti-CD3/anti-CAMA1, anti-CD3/anti-CD19, anti-CD3/MoV18, anti-neural cell ahesion molecule (NCAM)/anti-CD3, anti-folate binding protein (FBP)/anti-CD3, anti-pan carcinoma associated antigen (AMOC-31)/anti-CD3; BsAbs with one arm which binds specifically to a tumor antigen and one arm which binds to a toxin such as anti-saporin/anti-Id-1, anti-CD22/anti-saporin, anti-CD7/anti-saporin, anti-CD38/anti-saporin, anti-CEA/anti-ricin A chain, anti-interferon-α(IFN-α)/anti-hybridoma idiotype, anti-CEA/anti-vinca alkaloid; BsAbs for converting enzyme activated prodrugs such as anti-CD30/anti-alkaline phosphatase (which catalyzes conversion of mitomycin phosphate prodrug to mitomycin alcohol); BsAbs which can be used as fibrinolytic agents such as anti-fibrin/anti-tssue plasminogen activator (tPA), anti-fibrin/anti-urokinase-type plasminogen activator (uPA); BsAbs for targeting immune complexes to cell surface receptors such as anti-low density lipoprotein (LDL)/anti-Fc receptor (e.g. FcγRI, FcγRII or FcγRIII); BsAbs for use in therapy of infectious diseases such as anti-CD3/anti-herpes simplex virus (HSV), anti-T-cell receptor:CD3 complex/anti-influenza, anti-FcγR/anti-HIV; BsAbs for tumor detection in vitro or in vivo such as anti-CEA/anti-EOTUBE, anti-CEA/anti-DPTA, anti-p185^{HER2}/anti-hapten; BsAbs as vaccine adjuvants; and BsAbs as diagnostic tools such as anti-rabbit IgG/anti-ferritin, anti-horse radish peroxidase (HRP)/anti-hormone, anti-somatostatin/anti-substance P, anti-HRP/anti-FITC, anti-CEA/anti-β-galactosidase. Examples of trispecific antibodies include anti-CD3/anti-CD4/anti-CD37, anti-CD3/anti-CD5/anti-CD37 and anti-CD3/anti-CD8/anti-CD37. Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditonal production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature*, 305:537–539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.*, 10:3655–3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypepbde fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology*, 121:210 (1986).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science*, 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.*, 175: 217–225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each

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Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.*, 148(5):1547–1553 (1992). The leucine zipper pepbdes from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Nati. Acad. Sci. USA*, 90:6444–6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al, *J. Immunol*, 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tuft et al. *J. Immunol.* 147: 60 (1991).

(vii) Effector Function Engineering

It may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody. For example cysteine residue (s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron et al., *J. Exp Med.* 176:1191–1195 (1992) and Shopes, B. *J. Immunol.* 148:2918–2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctonal cross-linkers as described in Wolff et al. *Cancer Research* 53:2560–2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson et al *Anti-Cancer Drug Design* 3:219–230 (1989).

(viii) Antibody-Salvage Receptor Binding Epitope Fusions.

In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be desirable to modify the antibody fragment in order to increase its serum half life. This may be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (e.g. by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, e.g., by DNA or peptide synthesis).

The salvage receptor binding epitope preferably constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (e.g., of an IgG) and transferred to the CH1, CH3, or V_H region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain of the Fc region and transferred to the C_L region or V_L region, or both, of the antibody fragment.

In one most preferred embodiment, the salvage receptor binding epitope comprises the sequence (5' to 3'): PKNSSMISNTP (SEQ ID NO:4), and optionally further comprises a sequence selected from the group consisting of HQSLGTQ (SEQ ID NO:5), HQNLSDGK (SEQ ID NO:6), HQNISDGK (SEQ ID NO:7), or VISSHLGQ (SEQ ID NO:8), particularly where the antibody fragment is a Fab or $F(ab')_2$. In another most preferred embodiment, the salvage receptor binding epitope is a polypeptide containing the sequence(s) (5' to 3'): HQNLSDGK (SEQ ID NO:6), HQNISDGK (SEQ ID NO:7), or VISSHLGQ (SEQ ID NO:8) and the sequence: PKNSSMISNTP (SEQ ID NO:4).

(ix) Other Covalent Modifications of the Antibody

Covalent modifications of the antibody are included within the scope of this invention. They may be made by chemical synthesis or by enzymatic or chemical cleavage of the antibody, if applicable. Other types of covalent modifications of the antibody are introduced into the molecule by reacting targeted amino acid residues of the antibody with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues. Examples of covalent modifications are described in U.S. Pat. No. 5,534,615, specifically incorporated herein by reference. A preferred type of covalent modification of the antibody comprises linking the antibody to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Pat. Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

(x) Selecting Biologically Active Antibodies

Antibodies produced as described above may be subjected to one or more "biological activity" assays to select an antibody with beneficial properties from a therapeutic perspective.

The antibody may be screened for its ability to bind the antigen against which it was raised. In the case of an anti-CD18 antibody, as shown in the example below, the antigen binding properties of the antibody can be evaluated in a "MAC-1 capture assay". Briefly, the assay involves first coating ELISA plates with an anti-CD18 antibody that binds to a site of MAC-1 different from the anti-CD18 antibody of interest to capture a recombinant preparation of soluble MAC-1, followed by a wash, addition of the sample to be tested, wash, addition of goat HRP-labeled anti-human $F(ab')_2$ antibody and colonmetric detection of OPD substrate. The total amount of antibody may be measured by first coating an ELISA plate with a polyclonal anti-$F(ab')_2$ antibody, followed by addition of the sample, and then HRP-anti-$F(ab')_2$ and colorimetric detection of the HRP substrate, OPD. Specific activity is the ratio of the MAC-1 binding to total $F(ab')_2$ ELISA value.

In another embodiment, the affinity of the antibody may be determined by saturation binding; ELISA; and/or competition assays (e.g. RIA's), for example.

Also, the antibody may be subjected to other biological activity assays, e.g., in order to evaluate its effectiveness as a therapeutic. Such assays are known in the art and depend on the target antigen and intended use for the antibody.

Where the antibody binds CD18, examples of biological activity assays include a slide adhesion assay, phagocytosis assay, neutrophil binding assay and degranulation assay.

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The slide adhesion assay involves preincubating heparinized blood with various concentrations of anti-CD18 antibody, and then placing aliquots onto chambered glass slides. The slides are incubated at 37° C. to allow cells to adhere, nonadherent cells are gently washed off, the adherent cells are stained, and the average number of adherent cells per microscope field is determined for 30 fields.

For the phagocytosis assay, heparinized whole blood is obtained and the red blood cells are lysed. The cells are then incubated with opsonized BODIPY-labeled Staphylococcus aureus particles for 30 min at 37° C. The cells are then analyzed by FACS, and the fluorescence intensity in the neutrophil gate is determined as an indication of the extent of phagocytosis.

For determining binding of anti-CD18 antibody to neutrophils, whole blood is incubated with various concentrations of the anti-CD18 antibody. The cells are then stained with a FITC-conjugated goat anti-human $F(ab')_2$ antibody, the red blood cells are lysed, and the white blood cells are analyzed by FACS. The fluorescence intensity in the neutrophil gate is proportional to the extent of anti-CD18 antibody binding.

For a degranulation assay, neutrophils are isolated from whole blood and preincubated with anti-CD18 antibody. The cells are then stimulated with opsonized zymosan particles and allowed to stand at room temperature. The cellular supernatants are then collected, and degranulation is assessed either by specific ELISA (for myeloperoxidase or lactoferrin) or by enzyme assay (for elastase).

For other antibodies, examples of biological activity assays include tumor cell growth inhibition assays (as described in WO 89/06692, for example); antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated cytotoxicity (CDC) assays (U.S. Pat. No. 5,500,362); and agonistic activity or hematopoiesis assays (see WO 95/27062).

To screen for antibodies which bind to a particular epitope on the antigen of interest (e.g., those which block binding of the humanized H52 antibody of the example to CD18), a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping, e.g. as described in Champe et al., *J. Biol. Chem.* 270:1388–1394 (1995), can be performed to determine whether the antibody binds an epitope of interest.

B. Vectors, Host Cells and Recombinant Methods

For recombinant production of the antibody, the nucleic acid encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the monoclonal antibody is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence (e.g. as described in U.S. Pat. No. 5,534,615, specifically incorporated herein by reference).

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as Escherichia, e.g., *E. coli*, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella, e.g., *Salmonella typhimurium*, Serrafia, e.g, *Serratia marcescans*, and Shigeila, as well as Bacilli such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published Apr. 12, 1989), Pseudomonas such as *P. aeruginosa*, and Streptomyces. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X 1776 (ATCC 31,537), and *E coil* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

In additlon to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for antibody-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; Kluyveromyces hosts such as, e.g., *K. lactis, K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K. thermotolerans*, and *K. marxianus; yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070); Candida; *Trichoderma reesia* (EP 244,234); *Neurospora crassa*; Schwanniomyces such as *Schwanniomyces occidentalis*; and filamentous fungi such as, e.g., Neurospora, Penicillium, Tolypocladium, and Aspergillus hosts such as *A. nidulans* and *A. niger.*

Suitable host cells for the expression of glycosylated antibody are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed bySV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subloned for growth in suspension culture, Graham et al, *J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub et al., *Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243–251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., *Annals N.Y. Acad. Sci.* 383:44–68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transformed with the above-described expression or cloning vectors for antibody production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

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The host cells used to produce the antibody of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham et al., *Meth. Enz.* 58:44 (1979), Barnes et al., *Anal. Biochem.* 102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Pat. No. Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed cells, is removed, for example, by centrifugation or ultrafiltration. Where the antibody is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2, or γ4 heavy chains (Lindmark et al., *J. Immunol. Meth.* 62:1–13 (1983)). Protein G is recommended for all mouse isotypes and for human γ3 (Guss et al., *EMBO J.* 5:1567–1575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSET™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipiation are also available depending on the antibody to be recovered.

C. Preparation of the Formulation

After preparation of the antibody of interest as described above, the pharmaceutical formulation comprising it is prepared. The antibody to be formulated has not been subjected to prior lyophilization and the formulation of interest herein is an aqueous formulation. Preferably the antibody in the formulabon is an antibody fragment, such as an $F(ab')_2$, in which case problems that may not occur for the full length antibody (such as clipping of the antibody to Fab) may need to be addressed. The therapeutically effective amount of antibody present in the formulation is determined by taking into account the desired dose volumes and mode (s) of administration, for example. From about 0.1 mg/mL to about 50 mg/mL, preferably from about 0.5 mg/mL to about 25 mg/mL and most preferably from about 2 mg/mL to about 10 mg/mL is an exemplary antibody concentration in the formulabon.

An aqueous formulation is prepared comprising the antibody in a pH-buffered solution The buffer of this invention has a pH in the range from about 4.5 to about 6.0, preferably from about 4.8 to about 5.5, and most preferably has a pH of about 5.0. Examples of buffers that will control the pH within this range include acetate (e.g. sodium acetate), succinate (such as sodium succinate), gluconate, histidine, citrate and other organic acid buffers. The buffer concentration can be from about 1 mM to about 50 mM, preferably from about 5 mM to about 30 mM, depending, for example, on the buffer and the desired isotonicity of the formulation. The preferred buffer is sodium acetate (about 10 mM), pH 5.0.

An polyol, which acts as a tonicifier and may stabilize the antibody, is included in the formulation. In preferred embodiments, the formulation does not contain a tonicifying amount of a salt such as sodium chloride, as this may cause the antibody to precipitate and/or may result in oxidation at low pH. In preferred embodiments, the polyol is a nonreducing sugar, such as sucrose or trehalose. The polyol is added to the formulation in an amount which may vary with respect to the desired isotonicity of the formulation. Preferably the aqueous formulation is isotonic, in which case suitable concentrations of the polyol in the formulation are in the range from about 1% to about 15% w/v, preferably in the range from about 2% to about 10% whv, for example. However, hypertonic or hypotonic formulations may also be suitable. The amount of polyol added may also alter with respect to the molecular weight of the polyol. For example, a lower amount of a monosaccharide (e.g. mannitol) may be added, compared to a disaccharide (such as trehalose).

A surfactant is also added to the antibody formulation. Exemplary surfactants include nonionic surfactants such as polysorbates (e.g. polysorbates 20, 80 etc) or poloxamers (e.g. poloxamer 188). The amount of surfactant added is such that it reduces aggregation of the formulated antibody and/or minimizes the formation of particulates in the formulation and/or reduces adsorption. For example, the surfactant may be present in the formulation in an amount from about 0.001% to about 0.5%, preferably from about 0.005% to about 0.2% and most preferably from about 0.01% to about 0.1%.

In one embodiment, the formulation contains the above-identified agents (i.e. antibody, buffer, polyol and surfactant) and is essentially free of one or more preservatives, such as benzyl alcohol, phenol, m-cresol, chlorobutanol and benzethonium Cl. In another embodiment, a preservative may be included in the formulation, particularly where the formulation is a multdose formulation. The concentration of preservative may be in the range from about 0.1% to about

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2%, most preferably from about 0.5% to about 1%. One or more other pharmaceutically acceptable carriers, excipients or stabilizers such as those described in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980) may be included in the formulation provided that they do not adversely affect the desired characteristics of the formulation. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed and include; additional buffering agents; co-solvents; antoxidants including ascorbic acid and methionine; chelating agents such as EDTA; metal complexes (e.g. Zn-protein complexes); biodegradable polymers such as polyesters; and/or salt-forming counterions such as sodium.

The formulation herein may also contain more than one protein as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect the other protein. For example, where the antibody is anti-CD18, it may be desirable to provide a further anti-adhesion antibody, such as an anti-ICAM-1 or anti-CD11a antibody along with the anti-CD18 antibody in a single formulation. Alternatively, the anti-CD18 antibody may be combined with another anbinflammatory agent or a thrombolytic agent. Such proteins are suitably present in combination in amounts that are effective for the purpose intended.

The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtrabon through sterile filtration membranes, prior to, or following, preparation of the formulation.

D. Administration of the Formulation

The formulation is administered to a mammal in need of treatment with the antibody, preferably a human, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. In preferred embodiments, the formulation is administered to the mammal by intravenous administration. For such purposes, the formulation may be injected using a syringe or via an IV line, for example.

The appropriate dosage ("therapeutcally effective amount") of the antibody will depend, for example, on the condition to be treated, the severity and course of the condition, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, the type of antibody used, and the discretion of the attending physician. The antibody is suitably administered to the patent at one time or over a series of treatments and may be administered to the patent at any time from diagnosis onwards. The antibody may be administered as the sole treatment or in conjunction with other drugs or therapies useful in treating the condition in question.

As a general proposition, the therapeutically effective amount of the antibody administered will be in the range of about 0.1 to about 50 mg/kg of patent body weight whether by one or more administrations, with the typical range of antibody used being about 0.3 to about 20 mg/kg, more preferably about 0.3 to about 15 mg/kg, administered daily, for example. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques.

In the case of an anti-CD18 antibody, a therapeutically effective amount of the antibody may be administered to treat inflammatory disorders such as hemorrhagic shock, thermal injury (such as that resulting from burns), stroke (including ischemic and hemorrhagic stroke), and myocardial infarction. Where the antibody is an antIL8 antibody, the disorder may be an inflammatory disorder such as adult respiratory distress syndrome (ARDS), hypovolemic shock, ulcerative colits, and rheumatoid arthritis.

E. Articles of Manufacture

In another embodiment of the invention, an article of manufacture is provided comprising a container which holds the aqueous pharmaceutical formulation of the present invention and optionally provides instructions for its use. Suitable containers include, for example, bottles, vials and syringes. The container may be formed from a variety of materials such as glass or plastic. An exemplary container is a 3–20 cc single use glass vial. Alternatively, for a multidose formulation, the container may be 3–100 cc glass vial. The container holds the formulation and the label on, or associated with, the container may indicate directions for use. The article of manufacture may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

The invention will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the invention. All literature and patent citations are incorporated herein by reference.

EXAMPLE 1

This example describes an aqueous formulation comprising the antibody, recombinant humanized anti-CD18 antibody (rhuMAb CD18). RhuMAb CD18 having the amino acid sequence shown in FIG. 1A (heavy chain; SEQ ID NO:1) and FIG. 1B (light chain; SEQ ID NO:2) was created by humanization of the murine monoclonal antibody muMAb H52 (Hildreth et al. *J. Immunology* 134:3272–3280 (1985)).

The rhuMAb CD18 was produced recombinantly as described below. Plasmid pS 1130 was constructed to direct production of a rhuMAb CD18 precursor molecule with a leucine zipper domain in *E. coli*. The precursor is cleaved during the purification process by the protease pepsin to yield rhuMAb CD18. rhuMAb CD18 is an $F(ab')_2$ molecule composed of two different peptides (light and heavy chains) linked by disulfide bonds. Fusion of a yeast GCN4 leucine zipper dimerizabon domain to the C-terminus of an Fab' substitutes for the Fc region and allows for efficient $F(ab')_2$ production in *E. coli*. The GCN4 leucine zipper domains interact to form stable dimeric structures (parallel coiled coils) that hold the hinge region cysteine residues of two heavy chains together so that the two native interchain disulfide bonds can form. This results in formation of $F(ab')_2$ complexes that are covalently linked by disulfide bonds. The leucine zipper domains are later removed from the rhuMAb CD18 precursor during the purification process using the protease pepsin, which cleaves uniformly between the two leucine residues of the hinge. This results in the formation of the rhuMAb CD18 $F(ab')_2$ molecule.

Plasmid pS1130 (FIG. 20) is based on the well characterized plasmid pBR322 with a 2143 bp expression cassette (FIGS. 21A and 21B) inserted into the EcoRI restriction site. Plasmid pS1130 is resistant to both tetracycline and β-lactam antibiotics. The expression cassette contains a single copy of each gene linked in tandem. Transcription of each gene into a single dicistronic mRNA is directed by the *E. coli* phoA promoter (Chang et al. *Gene* 44:121–125 (1986)) and ends at the phage lamda to terminator (Scholtissek and Grosse *Nucleic Acids Research* 15:3185

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(1987)). Translation initiation signals for each chain are provided by *E. coli* STII (heat stable enterotoxin) (Picken et al. *Infection and Immunity* 42:269–275 (1983)) Shine-Dalgarno sequences. Translation of each chain begins with a 23 residue STII signal peptide that directs translocaton of the pepbdes across the cytoplasmic membrane into the periplasmic space. The STII signal peptide is then removed by the *E. coli* leader peptidase. The light and heavy chains fold into their native conformations after secretion into the periplasm and associate into the rhuMAb CD18 precursor, a covalently linked $F(ab')_2$. The leucine zipper domain is cleaved from the precursor during the purification process (see below) to yield rhuMAb CD18. The cell line used in the production of rhuMAb CDI8 is 49A5, derived from *E. coli* cell line W3110 (ATCC 27,325) as shown in FIG. **22**. The fermentation procedure takes place as shown in FIG. **23**. Production of rhuMAb CD18 precursor occurs when the medium becomes depleted in phosphate, typically 30–60 hours after inoculation.

Purification of rhuMAb CD18 precursor from the *E. coli* cell paste was as follows. Frozen cell pellets containing antCD18 precursor antibody, were dissolved in about 3 volumes of extraction buffer (120 mM MES, 5mM EDTA buffer, pH 6) heated to 30–40° C. This resulted in a suspension with a pH between about 5.4 and 6.5. This suspension was passed twice through a Gaulin homogenizer at 5500 to 6500 psi and kept below 20° C. with a heat exchanger. 5% polyethyleineimine (PEI) (wh), pH 6 was added to the homogenate to a final concentration of 0.2% PEI. The mixture was incubated for about one hour at 2–8° C. About one volume of extraction buffer (120 mM MES, 5 mM EDTA, pH 6) was added before the solids were removed by centrifugation at 15,280 g. The clear supernatant was conditioned to a conductivity of less than 3mohms by the addition of cold water. The conditioned supernatant was loaded onto a cation exchange column (ABX column; Mallinckrodt Baker, Inc., N.J., USA) equilibrated in 50 mM MES, pH 6.0. sodium citrate, pH 6.0. The cation exchange anti-CD18 precursor antibody pool was diluted with 50 mM MES, 36 mM sodium citrate, pH 4.0 to a concentration of approximately 2 g/L. The pool was then adjusted to pH 4 by addition of 2 M citric acid and flowed through a column containing immobilized pepsin (pepsin-CPG) previously equilibrated with 50 mM MES, 36 mM sodium citrate pH 4.0. Pepsin (Sigma, Mo., USA) was chemically coupled to controlled pore glass (CPG) by Bioprocess Ltd., NJ, USA; the CPG was activated with NalO4 followed by reduction of schiff base formation between CPG and pepsin using NaBH3CN). This procedure removed the zippers from the hinge region while leaving intact $F(ab')_2$. The effluent from the pepsin-CPG column was filtered directly in line through an anion exchange Sartobind Q filter (Sartorius, Goettingen, West Germany). The generated anti-CD18 $F(ab')_2$ antibody flows through the filter while pepsin and other negatively charge impurities bind strongly to the filter. The pool was diluted to give a conductivity of approx. 7 mohms by the addition of water and was then applied to a cation exchange column (SP Sepharose HP) equilibrated was 25 mM MES, 60 mM acetic acid, pH 4.0. The SP Sepharose column was washed with 25 mN MES, 75 MM sodium acetate pH 5.6 and eluted in a linear gradient of 75–110 mM sodium acetate in 25 mN MES pH 5.6. The pooled fraction from the SP sepharose column was diluted with the addition of 3.0M ammonium sulphate, 25 mM MES pH 6.0 at a ratio of 0.26 liters per liter of pool and was then passed through a hydrophobic interaction chromatography (HIC) column (phenyl sepharose FF-low substitution) previously equilibrated in 0.625 M ammonium sulphate, 25 nM MES pH 6.0. After loading, the column was washed with same buffer used in equilibration and the rhuMAb CD18 was eluted in 0.375M ammonium sulphate, 25 mN MES pH 6.0.

This resulted in purified rhuMAb CD18 for formulating as described below. Five aqueous formulations were evaluated for selection of a clinical formulation for use in, e.g., hemorrhagic shock. The formulations were as shown in Table 1 below.

TABLE 1

Matrix of formulations prepared for this study.

Formulation	Buffer	pH	TWEEN 20™ (0.01% v/v)	NaCl (140 mM)	Mannitol (4% w/v)	Trehalose (8% w/v)
F1	10 mM Acetate	5.0	+	+		
F2	10 mM Acetate	5.0	+			+
F3	10 mM Histidine	6.0	+	+		
F4	10 mM Histidine	6.0	+		+	
F5	10 mM Histidine	6.0	+			+

Two pH's were compared, pH 5 (sodium acetate) and pH 6 (histidine). At each pH, sodium chloride and trehalose were compared as tonicifiers. A histidine formulation with mannitol at pH 6 was also evaluated. The presence of a surfactant (e.g. 0.01% TWEEN 20™) was found to be required for stabilization against shaker-induced aggregation and for preventing adsorption to containers at protein concentrations below 1 mg/mL. The stability assays indicated that the trehalose formulations were generally superior to the others, with pH 5 showing slightly greater chemical stability than the pH 6 formulation due to reduced clipping. Kinetic data after 2 weeks and 5 weeks storage at 5, 30, and 40° C. were used for Arrhenius analysis and shelf-life prediction. Using cation exchange HPLC, the preferred formulation for rhuMAb CD18 was 10 mM sodium acetate, 8% trehalose whv, 0.01% TWEEN 20™, pH 5.0, with a predicted shelf life of 25–75 months (95% confidence intervals) at 5° C.

In designing antibody formulations, it may be useful to analyze the structural properties of the antibody to be formulated, but this is not necessary. RhuMAb CD18 consists of beta sheets of light (left side of the molecule in FIG.

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16) and heavy chains (right side of the molecule in FIG. 16) which are structurally stabilized each by two intramolecular disulfides and held together by two intermolecular disulfides (depicted as barrels at the bottom of FIG. 16). The CDRs (complimentarity determining regions) in the variable segments are oriented on the top of the molecule. Located within and close to the CDRs are a number of methionines, only one of which (Met 65) is fully exposed. Though Met 65 is buried in anti-CD18, it is near a histidine residue which, without being limited to any one theory, might promote metal induced oxidation of proximal methionine (Li et al. *Biochem.* 34(17):5762–5772 (1995)). Susceptibility to oxidation in the CDRs was assessed herein. It was found to be desirable to use a sugar as the tonicifler, rather than a salt, so as to minimize oxidation at low pH.

In assessing the potential deamidation or isomerization sites in this molecule, i.e. Asx-Gly or Asx-Ser (Asx indicates Asp or Asn) that are in hydrophilic and flexible regions (Clarke et al. in *Stability of Protein Pharmaceuticals. Part A. Chemical and Physical Pathways of Protein Degradation*, T. J. Ahern and M. C. Manning, Editors. 1992, Plenum Press: New York. p.2–29; and Kossiakoff, *Science*, 240:191–94 (1988)), six were found to be exposed and in fairly flexible regions (FIGS. 2A and 2B). Without being limited to any one theory, heavy chain motifs $MN^{84}S$, $KN^{55}G$, and light chain motifs $GN^{158}S$, $QD^{167}S$, $KD^{170}S$, are predicted to be most reactive; reactivity at Asn 84 is most likely to affect the binding activity of the antibody since it is near the CDR's. Asn 103 was found to be the primary route of degradation (via deamidation).

MATERIALS AND METHODS

The materials used in the following methods were as follows: glacial acetic acid 99.9% (MW 60.05); concentrated NaOH 18.94N (50% w/w; MW 40.0); concentrated HCl 37.8% (12.44N; MW 36.46); hisbidine (MW 155.16); NaCl (MW 58.44g); pharmaceutical grade trehalose dihydrate (MW 342.31); D-mannitol (MW 182.17); low peroxide TWEEN 20™; rhuMAb anti-CD18 (~1.3 mg/mL in MOPS, Na acetate, pH 6.9).

Buffer preparation and formulation set-up: Two liters of the following stock buffers were prepared for dialysis and formulation:

- 0.2 M Sodium Acetate, pH 5: 22.7 mL acetic acid, 17 mL 50% NaOH, made up to 2 L with Milli-Q water. Final pH 4.98. The solution was sterile filtered into 2 L Nalgene bottles and stored at 5° C.
- 0.2 M Histidine, pH 6: 62.06 g of Histidine, 20 mL concentrated HCl, made up to 2 L in a volumetric flask. Final pH 5.92. The solution was sterile filtered into 2 L Nalgene bottles and stored at 5° C.
- 2 M NaCl: 116.88 g NaCl, made up to 1 L with Milli-Q water. The solution was sterile filtered into 1 L Nalgene bottles and stored at 5° C.
- 18% Mannitol: 180 g mannitol, made up to 1 L with MilliQ water. The solution was sterile filtered into 1 L Nalgene bottles and stored at 5° C.
- 20% Trehalose: 400 g trehalose, made up to 2 L with Milli-Q water. Final pH was 6.4. The solution was sterile filtered into 2 L Nalgene bottles and stored at 5° C.
- 10% (v/v) TWEEN 20™: 10 mL of concentrated TWEEN 20™ was carefully removed and added to a 100 mL volumetric flask, diluted with Milli-Q water, and stirred until dissolved. Stored at 2–8° C. in the dark.

Formulation: Table 2 below shows the preparation of the buffers against which the starting protein bulk was dialyzed for this study. Approximately 40 mL of the bulk rhuMAb CD18 (~1 .3mg/mL protein concentration) was dialyzed (2 L for 4 hr at 5° C. and ~2 L overnight) to solutions prepared as shown in Table 2. The dialyzate was filtered with 0.2 μM Nalgene cellulose acetate filter units and stored under aseptic conditions for use as blank for UV and HPLC analysis.

TABLE 2

Summary of formulation preparation

Formulation		0.2 M buffer (mL)	18% Mannitol (mL)	20% Trehalose (mL)	1M NaCl (mL)	Approx. Water (mL)	pH	final pH	Made up to (mL)
F1	Acet/5	200	—	—	560	3000	5.07	5.07	4000
F2	Acet/5	200		1600		2000	5.17*	5.07	4000
F3	His/6	200	—	—	560	3900	6.03	6.03	4000
F4	His/6	200	889		—	2800	5.85*	6.01	4000
F5	His/6	200	—	1600		2000	5.97	5.97	4000

*Formulation F2 was adjusted with acetic acid buffer made by diluting 286 μL of glacial acetic acid and 200 mL of the 20% trehalose and mixed with Milli-Q water to a final 500 mL. This is 10 mM acetic acid with 8% trehalose. Approximately 250 mL was required to adjust the pH as required. Formulation F4 was adjusted with 0.5 mL of 50% NaOH.

Some of the remaining dialysis buffer was used to prepare formulation vehicles by adding TWEEN 20™, as shown in Table 3.

TABLE 3

Summary of preparation of formulation vehicles

Vehicle	Dialysis Buffer (mL)	10% TWEEN 20 ™ (μL)	Final Vol. (mL)
F1	41.4	41	41.4
F2	41.5	42	41.5
F3	42.4	42	42.4
F4	42.2	42	42.2
F5	41.4	41	41.4

Table 4 below shows the final appearance, pH, and protein concentration of the dialyzed protein solutions.

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TABLE 4

Summary of analysis of the dialyzed protein solutions

Formulation	OD280 diluted 1:1	Conc. = OD/1.32[c]	pH of protein solution	Vehicle pH	color/ appearance	Temp ° C.
F1	0.8239	1.248	5.09	5.09	CAC**	24.7
F2	1.1192	1.696	5.09	5.09	CAC	24.7
F3	0.84605	1.282	6.06	6.06	CAC	24.7
F4	0.89763	1.36	6.10	6.10	CAC	24.7
F5	1.116	1.69	5.91	5.91	CAC	24.7

*Extinction coefficient was later changed to 1.45 mL/mg/cm.
**CAC = clear and colorless.

These protein solutions were then diluted, using reserved dialysis buffer, to a final ~1 mg/mL protein concentration, and TWEEN 20™ was added to final 0.01% (v/v), as shown in Table 5.

TABLE 5

Summary of final formulation concentration adjustment and concentration determination

Form. no.	Dialysis buffer (mL)	10% TWEEN 20 ™ (μL)	Dialy. Protein (mL)	Starting Conc. (mg/mL)	Final Vol. (mL)	*Final OD280	*Final Conc. (mg/mL)	*OD 340–360
F1	8.16	41	33.2	1.248	41.4	1.3297	1.008	0.011
F2	17.0	42	24.5	1.696	41.6	1.3332	1.010	0.017
F3	9.26	42	33.1	1.282	42.4	1.3138	0.995	0.009
F4	11.2	42	31	1.36	42.2	1.3065	0.990	0.010
F5	16.9	41	24.5	1.69	41.4	1.3118	0.994	0.016

*Measured after filtration and filling.

The formulations were sterile filtered using 150 mL Nalgene filter units with cellulose acetate membranes in a sterile hood. The sterile formulated rhuMAb CD18 solutions were filled at 0.6 mL per 3 cc vial, labeled and stored at designated temperatures. Vehicle solutions were filtered and filled identically. Tables 6A (antibody formulations) and 6B (vehicle controls) below show the stability program for these formulations. Data presented here are up to 5 weeks storage.

TABLE 6A

CD18 antibody formulations

time	40° C. samples	30° C. samples	5° C. samples	−20° C. samples	−70° C. samples
t0			2	2	2
2 wk	2	2	2		
5 wk	2	2	2	2	2
8 wk	2	2	2		
12 wk	2	2	2	2	2
6 mo		2	2	2	2
9 mo–1 yr		2	2	2	2
	8 + 2 ext = 10	12	14	8	10 + 4 ext = 14

TABLE 6B

Vehicles

time	40° C. vehicles	30° C. vehicles	5° C. vehicles	−20° C. vehicles	−70° C. vehicles
t0			1	1	1
2 wk	1	1	1		
5 wk	1	1	1	1	2
8 wk	1	1	1		
12 wk	1	1	1	1	2
6 mo		1	1	1	2
1 yr		1	1	1	2
	4	6	6 + 2 = 8	4 + 2 = 8	8 + 2 ext

At the 2 week and 5 week timepoints, 2 vials from each formulation and storage temperature were frozen at −70° c. At the 5 week timepoint the frozen samples were thawed and batch analyzed together with control samples frozsen at −70° C. immediately after filling (t=0 weeks).

UV Spectroscopy: Samples were measured without dilution in a Hellma 2 mm wide, 1 cm path-length cell with raised bottom and black sides. Approximately 300 μL of sample was used for the measurements. The instrument was blanked with Milli-Q water. Samples were scanned from 200 to 400 nm with a 2 nm bandwidth and 1 second integration using a HP-8452A spectrophotometer.

Protein concentration was determined from the absorbance at 278 nm correcting for the absorbance at 320 nm using an extinction coefficient of 1.45 mL/mg/cm. The average value of absorbances in the region of A_{340} to A_{360} was followed as an indicator of scattering material.

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pH: An Orion 720A pH meter was used with a MicroElectrodes, Inc. MI-410 micro pH electrode. The electrode was calibrated with pH 4 and 7 standard buffers, re-checked every 10–15 samples, and recalibrated as required. The instrument was calibrated and samples were measured at room temperature (~23° C.).

RP-HPLC: RP-HPLC was carried out using a 7.5×75 mm TSK Phenyl-5PW column with 0.1% TFA as the A buffer, and 0.08% TFA in acetonitrile as the B buffer. An inline 0.5,M filter was used before the column was equilibrated at 10% B buffer and run at 55° C. Injections of 20 μL were made and eluted according to Table 7 below:

TABLE 7

Time (Minutes)	% B	Flow (mL/minute)
0	10	0.8
30	25	0.8
35	70	0.8
35.01	70	2
36	70	2
37	10	2
41	10	2

Data collected at 214 nm at 1 point per second were used for analysis.

SEC Assay: The mobike phase used was 200 mM NaCl, 50 mM sodium phosphate at pH 6.0. The runs were 40 minutes at a flowrate of 0.5 mL/minute with 40 μL injectons done at ambient temperature. A TSK G3000SWXL 30 cm×7.8 mm column with inline filter and a TSK guard column before the main column was used. Detection was at 214 nm.

Ion-Exchange HPLCAssay(IEX): IEX was run using 50 μL injections. The mobile phases were 33 mM each of MES/HEPESIPIPES, adjusted to pH 6.0 for A and pH 8.0 for B; C=Milli-Q water. The gradient shown in Table 8 below was used:

TABLE 8

Time (min)	% B	% C	Flow (mL/min)
0	0	50	1
3	0	50	1
15	20	50	1
50	50	50	1
50.1	100	0	2
51	100	0	2
51.1	0	50	2
60	0	50	2

The method used a 50×4.6 mm BakerBond carboxy-sulphon (CSx) cation exchange column with an inline 0.5 μM filter before the column, run at 40° C. Analysis was done with data obtained at 280 nm, due to the high buffer background.

Hydrophobic Interaction Chromatography (HIC): HIC was done on a 4.6×50 mm Baker Bond Butyl NPR column with inline 0.5 μM filter before the column. The running buffers were 2M ammonium sulfate in 20 mM Tris-HCl, pH 7 as the A buffer, and 20 mM Tris, pH 7 as the B buffer. The column was equilibrated at 50° C. with 10% buffer B at 1 mUminute and 10 μL injections were made. The gradient shown in Table 9 below was used for elution:

TABLE 9

Time	% B
0	10
1	10
35	100
37	100
37.1	10
42	10

Data were collected at 214 nm at 1 point per second.

SDS-PAGE: Pre-made 10 and 14% glycine SDS-PAGE gels (Novex, San Diego, Calif.) were used for analysis of the reduced and non-reduced samples, respectively. 10 μL of the 1 mg/mL sample diluted with 10 μL of 2×Tris-Glycine sample buffer (Novex) was heated 2 minutes at 95° C., as were high MW range and low MW range (Bio-Rad) markers diluted 1:20 in same sample buffer. Kaleidoscope MW marker was heated at 95° C., as well, with no dilution. When the samples cooled, 10 μL was loaded on the gels. The load of the MW marker solutions was 5 μL. The gel apparatus (Novex) was filled with 1×gel loading buffer (Media Services) and the gels were run at constant voltage of 125 mV and variable current for 2 hours. The gels were stained with the Novex Coomassic blue stain, overnight. The next day, the gels were washed with water for 2 hours and then were soaked in Gel Drying Solution (1×) (Novex) for ½ hour. The gels were then air-dried with the Novex cellophane system.

Gel Isoelectric Focusing Electrophoresis: IEF was done using Pharmacia 3.5 to 9.5 PAG plate gels. Pharmacia phosphoric acid and sodium hydroxide running buffers were used. Samples were prepared by mixing 10 μL of sample with 30 μL of 15% glycerol. Pharmacia broad range and high range IEF standards, as well as Serva Protein Test Mix 9 were run as pi markers. Pharmacia standards were prepared by mixing the standard (reconstituted with 0.5 mL of Milli-Q water) 1:1 with 15% glycerol. The Serva protein mix was reconstituted according to instructions and diluted 10-fold with 15% glycerol. On one gel, anti-HER2 MAb bulk (pI=8.8–9.0 calculated) was also run as a control. The HER2 sample (5 mg/mL) was diluted 40× with 15% glycerol. The gels were loaded with 20 μL of each sample adsorbed onto paper patches placed one third of the gel width from the positive electrode (the acid side). Gels were run at 10° C. with constant voltage set to 400 V for 30 minutes, after which the sample loading papers were removed and the gels were run for 1.5 more hours at 1500 V. Gels were fixed in TCA with sulfosalicylic acid for 30 minutes, and washed with water for 10 minutes (for the second gel) or IEF destain (for the third gel), and stained without washing out the TCA, as instructed in the Novex instructions (for the first gel). The first two gels were stained with Novex colliodal Coomassie according to the Novex instructions. The last gel was stained for 8 minutes with standard IEF Coomassie stain which was preheated to ~60° C., and then destained with IEF destain which had also been preheated to ~60° C. overnight. The gels were air-dried with the method described above for the SDS-PAGE gels.

Freeze/ThawStability: To demonstrate that rhuMAb CD18 would not degrade by freeze/thaw cycling in the formulations being tested, four vials of each formulation were subjected to three cycles of freezing and thawing. Two of the vials of each formulation were cycled between −70° C. and 2–8° C. and two were cycled between −20° C. and 2–8° C. Two vials of each formulation were held at 2–8 ° C. as controls. During each cycle the vials were allowed to

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remain in the freezer for at least 2 hours, until there was no visible liquid remaining in the vial. They were then transferred to 2–8° C. for at least 2 hours, until no solid remained. One vial of the acetate/trehalose, pH 5 formulation (F2) did not freeze at −20° C. on the first cycle, but froze immediately when placed on the shelf in a −70° C. freezer.

Shaker Studies: One vial of each formulation, and a vial of identically configured rhuMAb CD18 bulk, was placed horizontally into a rack on a Glas-Col 99A S60012 shaker. The shaker arm was adjusted to a radius of ~30 cm and the speed was set to 70 rpm. The samples were shaken for 24 hours at room temperature in the axial direction. A second vial of each formulation, and the bulk, was maintained at room temperature near the shaker, as a control. The samples were then analyzed by IEX, MAC-1 capture assay, and UV spectroscopy.

MAC-1 Capture (Receptor ELISA) and Total $F(ab')_2$ ELISA: rhuMAb CD18 is directed to the CD18 chain of MAC-1. The antigen binding property of rhuMAb CD18 can hence be evaluated by "MAC-1 capture assay". The assay involves coating of a 96 well mictrotitre plate first with a murine anti-CD18 antibody that binds to a site of MAC-1 different from rhuMAb CD18 (MHM23 MAb; Hildreth et al, *Eur. J. Immunol.* 13:202–208 (1983)) to capture a recombinant preparation of soluble MAC-1, followed by a wash with phosphate buffered saline (PBS), addition of the unknown sample, wash with PBS, addition of goat HRP-labeled anti-human $F(ab')_2$ antibody and colorimetric detection of OPD substrate. The total amount of antibody is measured by first coating an ELISA plate with a polyclonal anti-$F(ab')_2$ antibody, followed by addition of the sample, and then HRP-anti $F(ab')_2$ and calorimetric detection of the HRP substrate OPD. Assay diluents in both cases were 0.5% bovine serum albumin (BSA), 0.05% polysorbate 20, 1 mM $CaCl_2$, 1 mM $MgCl_2$ Specific activity was measured as the ratio of the MAC-1 binding to total $F(ab')_2$ ELISA value.

The samples were serially pre-diluted with assay diluent obtained to final dilutions of 20,000, 40,000, and 80,000 times in microtiter plates and Micronics tubes using multichannel pipettors. The dilution scheme used was as shown in Table 10 below. Samples of diluted rhuMAb CD18 bulk were included on each assay plate as controls. These bulk controls were diluted one extra 2×dilution to final 40,000, 80,000, and 160,000 times. The same dilutions were used for both assays. Samples were diluted the same day as they were thawed for the 5 week analysis and then frozen in the Micronics tubes prior to submission for assay the following day. The freezelthaw stability of rhuMAb CD18 diluted in assay diluent in these assays was verified.

TABLE 10

Step	Dilution (times)	Sample (μL)	Diluent (μL)
1, in 96 well plate*	10	10	90
2, in 96 well plate*	10	10	90
3, in 96 well plate*	10	10	90
4, in Micronics tubes	20	50	950
5, in Micronics tubes	2	500	500
6, in Micronics tubes	2	500	500

*Later studies were conducted by making all dilutions in Micronics tubes and vortexing them to mix; this improved reproducibility.

Kinetic Parameters, Arrhenius Calculations, and Statistics: The loss in the % main peak versus time was fitted to first order kinetics. The observed rate constants (K) at various temperatures were then fitted to the Arrhenius equation as shown below:

$$\ln K = -\Delta E/RT + C$$

where E is the activation energy (in cal/mol), R is the Universal gas constant (1.987 cal/mol/K), T is the absolute temperature (in Kelvins) and C is a constant. From the slope of a plot of ln K VS. 1/T, ΔE was calculated. The standard error (SE) of ln K was calculated using the formula $[(1/K)^2+(SE)^2]^{0.5}$. The reason for a large SE value for ln K at lower temperatures is the small value of the K which is in the denominator of the formula. A value of K extrapolated from the Arrhenius fit (±95% confidence interval) was used to predict the shelf life at 5° C. according to:

$$t_{90}(wk) = \ln 0.9/K$$

where K was expressed in $weeks^{-1}$. Confidence intervals were determined using the program SIGMAPLOT™.

RESULTS AND DISCUSSION

Freeze/Thaw stability: Table 11 below shows a summary of the data from analysis of the samples in the freeze/thaw study. No significant differences were seen between the different temperature groups. The observation that one vial of F2 did not freeze at −20° C. on the first cycle raised a concern that freezing of bulk in a jacketed stainless steel storage tank might be problematic. The coldest parts of the solution in these tanks reach only about −20° C., and therefore might not freeze completely. This was tested using formulation vehicle. The tank was observed to freeze solid within 6 hours of cooling, so no problem for bulk storage is predicted.

TABLE 11

Effect of three cycles of freezing and thawing to 5° C. on the Stability of rhuMAb CD18

Formulation	RP-HPLC Total Area	RP-HPLC % Main	SEC Total Area	SEC % Main	UV Conc. (mg/mL)	UV Average 340–360 nm	MAC-1 Capture Titer (μg/mL)	Specific Activity (MAC-1/UV)
F1 5° C.	10276 ± 25	88.0 ± 0.1	6401 ± 151	83.8 ± 0.4	0.949 ± 0.003	0.011 ± 0.001	1000 ± 32	1.05 ± 0.03
−20° C.	10341 ± 106	88.2 ± 0.1	6556 ± 73	83.2 ± 0.1	0.952 ± 0.002	0.012 ± 0.001	948 ± 28	1.00 ± 0.03
−70° C.	10328 ± 13	87.5 ± 0.01	6353 ± 63	83.0 ± 0.9	0.948 ± 0.005	0.013 ± 0.004	972 ± 15	1.03 ± 0.02
F2 5° C.	10537 ± 4	88.5 ± 0.2	6508 ± 18	81.7 ± 0.3	0.941 ± 0.006	0.017 ± 0.005	1069 ± 57	1.14 ± 0.06
−20° C.	10644 ± 107	87.3 ± 0.5	6290 ± 54	83.1 ± ± 0.2	0.941 ± 0.002	0.012 ± 0.001	1132 ± 27	1.20 ± 0.03
−70° C.	10503 ± 74	88.5 ± 0.2	6435 ± 120	82.5 ± 0.8	0.938 ± 0.000	0.009 ± 0.002	1064 ± 41	1.13 ± 0.04
F3 5° C.	10147 ± 50	87.9 ± 0.2	6311 ± 65	84.8 ± 0.3	0.926 ± 0.002	0.017 ± 0.001	1017 ± 77	1.10 ± 0.08
−20° C.	10050 ± 43	88.4 ± 0.00	6404 ± 39	85.6 ± 0.1	0.922 ± 0.007	0.012 ± 0.001	997 ± 28	1.08 ± 0.03
−70° C.	9953 ± 85	88.0 ± 0.2	6356 ± 52	83.9 ± 0.2	0.926 ± 0.002	0.017 ± 0.005	1021 ± 26	1.0 ± 0.03

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TABLE 11-continued

Effect of three cycles of freezing and thawing to 5° C. on the Stability of rhuMAb CD18

Formulation	RP-HPLC Total Area	RP-HPLC % Main	SEC Total Area	SEC % Main	UV Conc. (mg/mL)	UV Average 340–360 nm	MAC-1 Capture Titer (μg/mL)	Specific Activity (MAC-1/UV)
F4 5° C.	10397 ± 26	88.2 ± 0.1	6304 ± 148	84.5 ± 1.1	0.921 ± 0.000	0.010 ± 0.003	905 ± 18	0.98 ± 0.02
−20° C.	10126 ± 180	88.6 ± 0.03	6467 ± 13	83.5 ± 0.2	0.922 ± 0.004	0.014 ± 0.001	1004 ± 20	1.09 ± 0.03
−70° C.	10322 ± 51	88.1 ± 0.1	6327 ± 3	84.7 ± 1.1	0.924 ± 0.004	0.009 ± 0.007	982 ± 21	1.06 ± 0.03
F5 5° C.	10438 ± 81	88.0 ± 0.1	6418 ± 64	85.9 ± 0.3	0.930 ± 0.001	0.016 ± 0.004	1058 ± 39	1.14 ± 0.04
−20° C.	10443 ± 10	88.3 ± 0.1	6310 ± 11	86.5 ± 0.4	0.923 ± 0.004	0.016 ± 0.003	1010 ± 49	1.09 ± 0.05
−70° C.	10350 ± 135	88.3 ± 0.00	6340 ± 58	85.5 ± 0.1	0.923 ± 0.003	0.017 ± 0.006	1003 ± 22	1.09 ± 0.02

Mean ± SE of two vials are shown. No significant loss in monomer content or receptor binding was noticed.

UV Spectroscopy: Spectroscopic analysis of the samples was done to measure protein concentration as well as to measure light scattering as indicated by an increase in average absorbances in the range of 340 to 360 nm, or by a decrease in the ratio of the absorption maxima at 278 nm and the minima at 252 nm. FIGS. **3A** and **3B** show a summary of the UV data. The 40° C. samples of formulation F3 were very cloudy, containing white flocculent particles. These samples were microfuged for 5 minutes at 14000 RPM and re-measured after which the scatter was still relatively high. All other samples appeared clear and colorless ($A_{340-360}<0.02$, $A_{278}/A_{252}>2.4$). The change in A278/A250 was a more sensitive indicator of scattering than the average absorbances from 340 to 360 nm, since the latter showed no storage temperature related trend (FIG. **3A**), but the former showed a definite increase for the 40° C. samples (FIG. **3B**). The pH 5 formulations showed a smaller change in A278/A252 ratio at 40° C. than the pH 6 formulations in every case. The protein concentrations for the pH 5 formulations were only 2–3% greater than the pH 6 formulations (FIG. **4**).

pH: All formulation vehicles except F4 showed a slight increase (~0.1 units at 40° C.) in pH, compared with −70° C. controls (Table 12 below). Both pH 5 formulations showed a greater pH increase in the active samples than in the vehicles. None of the pH 6 formulations showed this difference between active and vehicle samples. In fact, the pH increase seen in the F3 and F5 vehicles was not seen in the active samples. The pH of F4 decreased slightly at higher storage temperature. The pH increase in the pH 5 samples could be explained by a loss in acetic acid concentration due to diffusion through the topper, and is not expected to be significant at 5° C.

TABLE 12

Formulation pH after 5 weeks storage.

Sample	pH of Vehicle	pH of Active (both vials shown)
F1 at −70° C.	4.892	5.009/5.026
F1 at −20° C.	4.894	5.028/5.035
F1 at 5° C.	4.947	5.077/5.082
F1 at 30° C.	5.040	5.154/5.164
F1 at 40° C.	5.045	5.171/5.171
F2 at −70° C.	5.037	5.179/5.089
F2 at −20° C.	5.041	5.096/5.101
F2 at 5° C.	5.098	5.151/5.153
F2 at 30° C.	5.134	5.192/5.213
F2 at 40° C.	5.136	5.242/5.215
F3 at −70° C.	6.123	6.164/6.154
F3 at −20° C.	6.162	6.155/6.138

TABLE 12-continued

Formulation pH after 5 weeks storage.

Sample	pH of Vehicle	pH of Active (both vials shown)
F3 at 5° C.	6.160	6.130/6.086
F3 at 30° C.	6.204	6.138/6.090
F3 at 40° C.	6.214	6.098/6.075
F4 at −70° C.	5.964	6.014/6.016
F4 at −20° C.	6.017	6.050/6.019
F4 at 5° C.	6.021	6.004/5.988
F4 at 30° C.	5.928	5.914/5.897
F4 at 40° C.	5.880	5.849/5.854
F5 at −70° C.	5.653	5.751/5.754
F5 at −20° C.	5.729	5.761/5.766
F5 at 5° C.	5.727	5.768/5.777
F5 at 30° C.	5.797	5.843/5.833
F5 at 40° C.	5.812	5.822/5.831

The pH 5 formulations showed a small increase in pH at higher storage temperatures for the vehicles and active samples. Formulations F3 and F5 showed increasing pH in the vehicles with higher storage temperature, but the active samples did not show this increase. Formulation showed a slight reduction in pH.

SEC: FIGS. **5A** and **5B** show the chromatograms for formulations F2 (FIG. **5A**) and F5 (FIG. **5B**). The main peak of rhuMAb CD18 eluted at 18.6 minutes, corresponding to an apparent MW of ~70,000 D. There were two major impurity peaks that eluted after the main peak, and the one at 20.9 minutes increased considerably with increasing time and storage temperature. The increase in the peak at 20.9 minutes was much more pronounced in F5 (FIG. **5B**) compared to F2 (FIG. **5A**), without being limited to any one theory, most likely due to the higher pH of F5. FIGS. **6A** and **6B** show graphs of the total peak area and the % main peak for all of the formulations after 0, 2 and 5 weeks of storage at 5, 30 and 40° C. The total peak areas for formulations F3 and F4 were significantly lower than the other three formulations.

Since the lower pH formulations showed the greatest stability, rate data from the % main peak of the SEC chromatogram were calculated and fitted to an Arrhenius plot. Data for F2 is shown in FIG. **7**. This plot contained only two useful points since the rate constant at 5° C. had a large standard error. The activation energy obtained from these data (19±6 kcal/mol) was very similar to the one calculated from the IEX data (see FIG. **15**) even without the 5° C. datum. The 95% confidence intervals to the curve fit predict a shelf-life from 6 to 60 years at 5° C.

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HIC: This assay was performed at a column temperature of 50° C. rather than 55° C. to improve protein recovery (from ~30% to nearly 90%), unfortunately at the expense of loss in resolution. HIC analysis of the stability samples showed an increase in a poorly resolved peak on the leading shoulder of the main peak, as well as an increase in a 17.5 min peak with increasing storage time and temperature for all formulations (FIG. **8**). The shoulder was in a position that would be consistent with an oxidized form of the protein, though in preliminary assessment no peaks corresponding to an oxidized species appeared by peptde mapping. F2 showed the smallest increase in this leading peak (FIG. **9**). A trend towards reduced recovery (total area) was also seen with increasing storage temperatures, with all formulations showing the same total area at 40° C. (FIG. **10**). Formulations F2 and F5 showed the least change in total area at 5 and 30° C. relative to the starting −70° C. sample. This method was not stability indicating because the resolution between the degradation peak at 17.5' and the main peak is too low for accurate quantitation, and the recovery from the method is poor.

RP-HPLC: The only change observed by RP-HPLC was an increase in a peak eluting at about 25 minutes, with a corresponding reduction in the main peak area, as the storage temperature increased (FIGS. **11** and **12**). The magnitude of this change was similar to that of the lower MW species seen in SEC. Based on the relative position of this peak and its area, it may correspond to the low MW species. Formulation F2 showed the least change in the % main peak area with increasing temperature. Samples reduced with DTT were also run on RP-HPLC to increase the chance that an oxidized form might be resolved from one of the separated smaller subunits of the antibody. No difference was seen between the stored samples and the −70° C. control samples with this method.

The Arrhenius plot of the RP-HPLC kinetic data for formulation F2 gave a similar activation energy (21±1 kcal/mole) to the SEC and IEX results (19–20 kcal/mole) (FIG. **13**). The shelf-life indicated by the 95% confidence intervals to the curve fit is 4 to 150 years at 5° C.

IEX: By this assay, earlier eluting peaks with retention times of 18.4 and 26.6 minutes increased with increasing storage temperature. As shown in FIGS. **14**A and B which represent formulations F2 and F5, respectively, there was a more pronounced increase of these peaks in F5, compared to F2. Also the peak at 19.5 minutes became broader at higher temperatures. Based upon chromatographic changes seen upon exposure of samples to pH 11 (see below), this method should readily detect changes such as deamidation and/or disulfide scrambling, both of which can be induced at alkaline pH.

Table 13 below shows the first order rate constants for the 5 formulations at 5, 30, and 40° C.

TABLE 13

First order rate constants ($\kappa \pm SE$; $n = 2$) for all formulations based on % main of IEX

Formulation	5° C.	30° C.	40° C.
F1	0.00064 ± 0.0021	0.0083 ± 0.0014	0.0299 ± 0.0023
F2	0.0065 ± 0.0023	0.0091 ± 0.0025	0.0245 ± 0.0040
F3	−0.0004 ± 0.0008	0.0143 ± 0.0010	0.0432 ± 0.0019
F4	−0.00066 ± 0.00073	0.0137 ± 0.00033	0.0413 ± 0.00022
F5	−0.00010 ± 0.00082	0.0129 ± 0.00095	0.0439 ± 0.0017

Formulations F1 and F2 are clearly better than the other formulations, implying that the major degradation process is base catalyzed. An Arrhenius plot based on the % main peak area predicted an activation energy of 19.8±9.9 kcal/mole for F2 and a t_{90} of 50±25 months (95% confidence intervals) at 5° C. (FIG. **15**). Receptor binding activity: Table 14 below shows the specific activity for each temperature and formulation after 5 weeks storage. The specific activity was calculated by dmding the MAC-1 capture assay concentration by the total $F(ab')_2$ ELISA concentration. The standard error shown is for n=2 and accounts for the total propagated error from the two different assays for 2 vials at 3 dilutions each. There was a clear trend for all formulations towards a lower specific activity at higher storage temperatures (~10–15% loss at 40° C.); all formulations showed about the same extent of loss at 40° C. relative to −70° C. The p value from one way ANOVA analysis of the 40° C. data for the different formulations is 0.98, so the 40° C. means would not be considered significantly different from each other, within 95% confidence. An unpaired t-test of the 40° C. data versus the 30° C. data shows that the difference is significant (p=0.044) and the lower temperatures are even more significantly different by this method (p<0.02).

TABLE 14

Storage	F1		F2		F3		F4		F5	
Temp (° C.)	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
−70	0.976	0.098	0.906	0.140	1.008	0.112	0.952	0.116	0.918	0.433
−20	0.978	0.123	0.903	0.157	0.946	0.152	0.871	0.072	0.865	0.209
5	0.982	0.157	0.898	0.177	1.023	0.166	0.919	0.203	0.886	0.350
30	0.923	0.067	0.878	0.189	0.949	0.076	0.868	0.109	0.885	0.340
40	0.866	0.113	0.817	0.227	0.904	0.115	0.817	0.093	0.772	0.198

IEF: The effect of storage for 5 weeks at different temperatures was assayed by isoelectric focusing electrophoresis (IEF) for formulations F2 and F5. The major band appeared to have a pI of ~8.8. Three main components at pI's ~8.75 (second most intense band), ~8.6, and ~8.4 were also seen, which increase slightly with storage, particularly at pH 6. The 30 and 40° C. samples showed conversion to the more acidic bands. For the 40° C. samples, the bands at pI 8.8 and

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8.75 had approximately equal intensity and there was no discernible difference between the formulations in this regard. There were at least two more acidic bands (pI's ~8.6 and ~8.4), which had at least 4-fold lower intensity than the two most basic bands. These bands did not seem to change in intensity with storage.

SDS-PAGE: The effect of storage temperature on the stability of rhuMAb CD18 formulations F2 and F5 (5 weeks) was assayed by SDS-PAGE (under reduced and nonreduced conditions). The rhuMAb CD18 appeared at a MW of ~120 kD on the non-reduced gel. In the starting bulk there were several minor bands at higher molecular weight than the main band which may represent rhuMAb CD18 with various portions of the leucine-zipper segment sill attached. Several percent of this impurity is known to be present in this bulk preparation. There were several bands of lower MW than the main band, but only one of these bands seemed to change in any of the formulations, or with temperature. The band at around 45 kD increased with temperature and seemed slightly more intense in pH 6 than in pH 5 formulations. This observation was consistent with SEC data. The band just above the ~45 kD band on the non-reduced gels corresponded in apparent molecular weight with the band seen on the reduced gels and may represent a non-reducing contaminant protein. All species converted to two bands, corresponding to light and heavy chain, upon reduction with dithiothreitol, suggesting the absence of any proteolytic cleavage. MALDI-TOF MS (Yates, *J. Methods Enzymol.*, 271:351–377 (1996)) confirmed formation of fragments that were the size of Fab'.

Formulation pH and protein concentration: The effect of pH on the conformation of rhuMAb CD18 was investigated using fluorescence spectroscopy (FIG. **17**). The protein appeared to lose tertiary structure below pH 3, but is unchanged in the range of pH 3 to 8.

Based on the above observation, the effect of pH and protein concentration on the stability of rhuMAb CD18 was then investigated using a central composite design protocol. Formulations containing 10 mM Na citrate, 8% trehalose, 0.05% TWEEN 20™ containing one of the following conditions were prepared: 0.5 mg/mL (pH 4.5), 5 mg/mL (pH 3, 4.5, 6), 10 mg/mL (pH 4, 5), 25 mg/mL (pH 4.5). Samples were placed at 40° C. and −70° C., and analyzed after 2 month by IEX (FIG. **18A**) and SEC (FIG. **18B**). Again, pH 5 was found to be the most stable condition for the protein, irrespective of protein concentration.

Long Term Stability of rhuMAb CD18 Formulation: To address the long term stability of the F2 formulation, duplicate samples in 10 mM Na acetate, 8% trehalose, 0.01% TWEEN 20™, pH 5 were prepared in 3 cc glass vials and placed on stability at the indicated temperatures. Analysis by SEC (FIG. **19A**) and MAC-1 binding (FIG. **19C**) indicates no change in the size distribution, aggregation state, or bioactivity of the protein for up to 43 weeks at 5° C; stability up to ~1 mo at 30° C. is indicated. IEX shows formation of acidic peaks (~1% after 43 weeks) that were identified to be deamidation by peptde mapping/MS (FIG. **19B**).

CONCLUSIONS

Preliminary data suggested that the primary reaction in the purified rhuMAb CD18 was cleavage to species approximately half the MW of the starting material, and that this reaction, as well as generation of acidic peaks on IEX and earlier eluting peaks on HIC and RP-HPLC, were all minimized at pH 5 (compared to pH 6), and in trehalose (compared to salt or mannitol). The smaller MW species may have been formed either by proteolytic cleavage and/or by disulfide scrambling, both of which may be enhanced at higher pH's. Reduction of the control and degraded samples led to only the light and heavy chains, consistent with either the cleavage to Fab, or mixed disulfide formation between heavy and light chains.

RhuMAb CD18 appeared to be least stable in salt formulations; at pH 5 a larger aggregate (equivalent to a trimer) appeared and at pH 6 precipitation was noted at 40° C. Without being bound to any one theory, one possible explanation is that NaCl does not provide hydrogen bonding, as sugars and mannitol do, which could potentially prevent self-association of this antibody at higher temperatures.

Based on the above preliminary data from the primary assays (IEX, SEC, and UV), the preferred aqueous formulation for rhuMAb CD18 is 10 mM sodium acetate, 8% trehalose wN, 0.01% TWEEN 20™, pH 5.0. The shelf-life predicted from an Arrhenius fit to the first order rate constants (IEX data) is 1.7 to 4 years at 5° C. (95% confidence intervals). This formulation can be prepared by mixing 0.573 mL glacial acetic acid, 0.403 mL concentration NaOH, 80 g trehalose, 1 mL of a 10% TWEEN 20™ solution and making up to 1 L with MilliQ water (pH 5.0±0.1 at the 2 L scale). Conductivity of this formulation was found to be 502±10% microSiemens/cm using a Radiometer-Copenhagen CDM-83 with a CDC 314 probe, and density was 1.017 g/mL.

EXAMPLE 2

This example describes the production of a stable aqueous multdose formulation comprising a recombinant humanized anti-CD20 antibody, rhuMAb CD20. Acetate (pH 5) formulations stored at 40° C. for one month demonstrated greater stability than those samples formulated in histidine (pH 5 or 6). The histidine formulations after accelerated temperature storage became very opalescent and yellow in color. A buffering capacity of 10–30 mM acetate was sufficient to maintain the pH at 5.0. The effective amount of tonicity modifier needed to stabilize the antibody against freeze or thermal induced aggregation was compared using sodium chloride (NaCl) or trehalose. Trehalose was found to protect the formulation from freeze induced aggregation, particularly at levels >134 mM (500:1 molar ratio). The trehalose formulations (67–270 mM) were much more effective than NaCl in stabilizing formulations placed at 40° C. as evidenced by the clarity of the solution. These results led to the development of a stable prototype liquid multidose formulation comprising 40 mg/mL rhuMAb CD20, 25 mM acetate, 150 mM trehalose, 0.9% benzyl alcohol, 0.02% polysorbate 20 at pH 5.0 that has a minimum shelf life of two years storage at 2–8° C.

MATERIALS AND METHODS

RhuMAb CD20: The bulk material used in all studies was composed of 27 mg/mL rhuMAb CD20 in 50 mM Tris and 100 mM sodium chloride at pH 7.5 or 2.7 mg/mL rhuMAb CD20 in 25 mM Tris and 50 mM sodium chloride at pH 6.0. The bulk was stored aseptically at 2–8° C. in the absence of light.

Buffer exchange: The exchange of the bulk rhuMAb CD20 into different test buffers was done by dialysis at 2–8° C. using Spectra/Por® 7 membranes (MWCO=8 Kda) which were rinsed thoroughly with deionized water before use. One volume of antibody was dialyzed against a minimum of 10 volumes of the appropriate test buffer. This process was repeated three to four times within a one day period. To obtain the final concentration of 40 mg/mL, the

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antibody was further concentrated using an Amicon UF/DF cell containing a YM30 membrane (MWCO=30 KDa). Due to the lower starting concentration of the 2.7 mg/mL material, this material was first concentrated to ~40 mg/mL and then dialyzed against the appropriate test buffer. After the target concentration was reached and the buffer exchange completed, trehalose, benzyl alcohol and polysorbate 20 were added to the final concentrations described in Tables 15 to 17 below. The liquid multdose C2B8 candidate formulation was 40 mg/mL rhuMAb CD20 formulated in 25 mM acetate, 150 mM trehalose, 0.9% benzyl alcohol and 0.02% polysorbate 20 at pH 5.0. The formulation was then sterile filtered through a 0.2μ membrane and the concentration was determined by UV spectrophotometric scan. A 0.5 ml of each final formulation was then filled into sterile 3 cc glass vials, stoppered with teflon faced grey butyl rubber stoppers and then capped with crimp seals.

TABLE 15

pH and buffer species comparison study

Concentration of rhuMAb CD20 (mg/mL)	Concentration of buffer species (mM)	pH	Storage Temperature (° C.)
40	50 mM Acetate	5.0	2–8, 40, 50
40	50 mM Histidine	5.0	2–8, 40, 50
40	50 mM Histidine	6.0	2–8, 40, 50

All formulations contain 150 mM trehalose, 0.9% benzyl alcohol and 0.02% polysorbate 20.

TABLE 16

Acetate buffering capacity study

Concentration of rhuMAb CD20 (mg/mL)	mM Acetate	Storage temperature (° C.)
40	10	2–8, 40
40	15	2–8, 40
40	20	2–8, 40
40	25	2–8, 40
40	30	2–8, 40

All formulations contain 150 mM trehalose, 0.9% benzyl alcohol and 0.02% polysorbate 20 at pH 5.0.

TABLE 17

Effective ratio of tonicity modifier: rhuMAb CD20 study

Tonicity modifier	Molar ratio of sugar/salt:rhuMAb CD20	mM trehalose or sodium chloride
Trehalose	0	0
Trehalose	250:1	67
Trehalose	500:1	134
Trehalose	1000:1	267
Sodium Chloride	500:1	134
Sodium Chloride	1000:1	267

A). All formulations contained 40 mg/mL rhuMAb CD20, 20 mM acetate, 0.9% benzyl alcohol and 0.02% polysorbate 20 at pH 5.0.
B). A freeze-thaw comparison study was completed for the trehalose formulations. The stability of all formulations at 2–8° and 40° C. was conducted in parallel.

SEC HPLC: Samples were diluted to 10 mg/mL with formulation buffer before being assayed. The method uses a TSK G3000 SWXL column (TosoHaas) with a mobile phase consisting of 0.2M potassium phosphate, 0.25M potassium chloride, pH.7. The socratic flow rate is 0.5 mLlmin with a total run time of 30 minutes. The amount of protein injected is 200 μp and the UV absorbance at 280 nm is used as the mode of detection.

HIC HPLC: Samples were diluted to 10 mg/mL with formulation buffer before being assayed. The antibody was digested with carboxypeptidase and papain prior to analysis of the final fragments. The method uses a TSK-GEL butyl-NPR (4.6x35 mm) column. The temperature of the column is controlled at 35° C. during the assay. Elution of the antibody fragments is induced by changes in the ammonium sulfate gradient. The total run time of the assay is forty minutes and the flow rate is 1 ml/min. A protein load of 5–10 μg is injected and the detection is monitored by UV absorbance at 214 nm.

UV spectrophotometric scan: For protein concentration determination, samples were accurately diluted 1:100 with formulation buffer. The absorbance at 280, 320 and 350 were read with an Hewlett Packard 8451A diode array spectrophotometer against the same formulation buffer as blank. The protein concentration was calculated by subtracting A_{320} from A_{280} and dividing by an extinction coefficient of 1.7.

For turbidity evaluaton, samples were scanned without dilution on an Hewlett Packard 8451A diode array spectrophotometer and the average absorbance in the range of 340–360 nm was determined. Water was used as the blank.

Accelerated stability studies: The vials were stored upright in temperature-controlled rooms or incubators at 2–8°, 30°, 40° or 50° C. Two to three vials were removed at finite times and the protein degradation monitored by the stability indicating assays (SEC HPLC, HIC HPLC, UV spectrophotometric scan to determine protein concentration and turbidity, as well as pH measurement). In addition, samples were subjected to the complement dependent cell cytotoxicity assay as described below to assess bioactvity.

Freeze-thaw studies: Samples were exposed to a minimum of two hours of freezing at -70° C. followed by a room temperature thaw (<45 minutes). Each cycle was composed of one freeze followed by one thaw excursion. Three vials were removed per formulation after one, three and five consecutive freeze-thaw cycles and the stability monitored by SEC HPLC and UV spectrophotometric scan to measure the turbidity and protein concentration.

Complemnent Dependent Cytotoxicity Assay (CDC): The bioactivity of the stability samples was determined by the CDC assay described in Gazzano Santoro et al., *J. Immunol. Meth.* 202:163–171 (1997), except that human complement (rather than rabbit complement) was used. The percent bioactvity of the test sample was determined as follows,

$$\%\ \text{bioactivity} = \frac{[(CDC\ \text{assay mg/mL of sample}/\ \text{protein concentration of sample})]}{[(CDC\ \text{assay mg/mL of reference}/\ \text{protein concentration reference})]} \times 100$$

The protein concentration of the test sample and reference control were determined by UV spectrophotometric scan.

RESULTS AND DISCUSSION

pH and buffer species: Decreasing the pH from 7.5 to 5.0 of 40 mg/mL rhuMAb CD20 formulated in histidine and trehalose virtually eliminated oxidation of the antibody even after two months storage at 40° C. (FIG. **24B**). There is also a decline in the rate of aggregation but the difference below pH 6.5 is slight (FIG. **24A**). Further reduction in aggregation rate may require a decrease in the protein concentration. A stable formulation appears to require a pH in the acidic range.

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To differentate between the effect of buffer species and the effect of pH, histidine (5 or 6) and acetate (5), multdose formulations were compared after storage at 2–8°, 40° or 50° C. The samples were stored at 50° C. as a way of quickly determining the relative stability between the formulations with the caveat that the degradation observed was not necessarily predictive of that seen at 2–8° C. storage.

The visual clarity of the formulations and the turbidity as measured by UV spectrophotometric scan (340–360 nm) after four (50° C.) and eight (2–8°, 40° C.) weeks storage is described in Tab Both histidine formulations were more opalescent than the acetate at all temperatures studied. After two weeks storage at 50° C., the histdine at pH 5 had formed a solid opaque gel while the sibling formulation at pH 6 was visually cloudy and yellow in color by four weeks. The histidine formulations stored at 40° C. also turned yellow. Without being bound to any one theory, the color formation is likely due to the oxidation of histidine and is more apparent in this study due to the high concentration of histidine used (50 mM). No differences were observed at 2–8° C. storage relative to the initial timepoint.

TABLE 18

The effect of buffer species and pH on the appearance and clarity of 40 mg/mL rhuMAb CD20 multidose formulations containing 50 mM acetate or histidine, 150 mM trehalose, 0.9% benzyl alcohol and 0.02% polysorbate 20 at pH 5 or 6.

Formulation	Temperature	Appearance	Turbidity Avg. O.D. 340–360 nm
T = 8 weeks			
Acetate pH 5		Clear	0.051 ± 0.001
Histidine pH 5	2–8° C.	Clear	0.065 ± 0.0002
Histidine pH 6		Clear	0.068 ± 0.001
Acetate pH 5	40° C.	Opalescent	0.16 ± 0.002
Histidine pH 5	40° C.	Light yellow, Opalescent	0.28 ± 0.015
Histidine pH 6	40° C.	Light yellow, Opalescent	0.36 ± 0.026
T = 4 weeks			
Acetate pH 5	50° C.	Very Opalescent	0.61 ± 0.007
Histidine pH 5	50° C.	Firm opaque gel	N/D[1]
Histidine pH 6	50° C.	Cloudy, yellow solution	1.30 ± 0.20

[1]The turbidity was not determined (N/D) due to the gelation of the sample.

The stability was also monitored by HIC and SEC HPLC methods. After eight weeks storage at 40° C., the acetate pH 5 formulations were unchanged while the histidine pH 6 formulations had a 18 percent reduction in unoxidized Fc relative to the initial bmepoint (Table 19). The percentage monomer decreased in all formulations stored at 40° C. with the histidine pH 6 being slightly more stable. This reduction was attributed to the formation of a high molecular weight aggregate(s) eluting at the void volume, a lagging shoulder on the monomer peak and lower molecular weight species. The protein concentration and pH were also measured, except in the case of gelation, and no changes were observed over the duration of the study.

TABLE 19

The effect of buffer species and pH on the percentage unoxidized Fc and percentage monomer of 40 mg/mL rhuMAb CD20 multidose formulations containing 50 mM acetate or histidine, 150 mM trehalose, 0.9% benzyl alcohol and 0.02% polysorbate 20 at pH 5 or 6.

Formulation	Temperature	% Unoxidized Fc (HIC HPLC)	% Monomer (SEC HPLC)
T = 0			
Acetate pH 5	2–8° C.	93.9	99.4
Histidine pH 5	2–8° C.	99.1	99.4
Histidine pH6	2–8° C.	99.3	98.5
T = 8 weeks			
Acetate pH 5	40° C.	96.5	90.4
Histidine pH 5	40° C.	87.0	87.4
Histidine pH 6	40° C.	81.7	93.9

Since the acetate pH 5 formulation had only a slightly higher aggregation rate, did not turn yellow upon storage at high temperatures and had the greatest clarity under all conditions studied, it was chosen as the buffer species and pH of choice for all subsequent liquid C2B8 multidose formulation screens.

Amount of buffering species: The amount of acetate which maintained the pH of a 40 mg/mL C2B8 multidose formulation at 5.0 was determined. Summarized in Table 20 is the effect on pH as the acetate buffer concentration was increased from 10 to 30 mM. At two and four weeks there appeared to be a slight advantage in staying above 15 mM acetate, although this did not hold true upon long term storage. No change was seen in the pH in any of the test formulations studied after one years storage at 2–8° C. A range of 10 to 30 mM acetate is sufficient to maintain the pH at 5.0.

TABLE 20

The effect of acetate buffer concentration on maintaining the pH of liquid rhuMAb CD20 multidose formulations at 5.0. The formulations were composed of 40 mg/mL rhuMAb CD20, 10 to 30 mM acetate, 150 mM trehalose, 0.9% benzyl alcohol, 0.02% polysorbate 20 at pH 5.0.

mM	pH (weeks)			
Acetate	0	2	4	56
2–8° C.				
10	5.03	5.12	5.11	5.00
15	5.07	5.12	5.14	5.06
20	5.04	5.04	5.04	5.00
25	5.01	5.00	5.02	4.98
30	5.01	5.00	5.02	4.98
40° C.				
10		5.12	5.14	
15		5.10	5.17	
20		5.08	5.10	
25		5.05	5.05	
30		5.03	5.05	

Ratio of tonicity modifier: protein: The addition of trehalose was beneficial to the stability of rhuMAb CD20 multidose formulations after multiple freeze/thaw cycles (FIG. 25). A decrease in the formation of soluble aggregates was observed as the concentration of trehalose in the formulation was increased. At a ratio of 500 moles trehalose:1 mole C2B8 (134 mM trehalose), the percentage of aggregate

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formed was only 0.5% after five freeze-thaw cycles while the control formulation containing no isotonicity modifier formed 4% aggregate. Having at least a 500 fold molar excess of trehalose in the liquid C2B8 formulation offered sufficient protection against freeze induced aggregation.

The ability of trehalose to stabilize rhuMAb CD20 during accelerated temperature storage was studied and compared to sodium chloride.

FIG. **26** describes the effect of excess molar ratios of trehalose or sodium chloride on the clarity of test formulations stored at 40° C. Sodium chloride is deleterious to the stability. After two weeks storage at 40° C., the 500:1 ratio sample had an O.D. of 0.44. At a 1000:1 molar ratio, the sodium chloride containing formulation separated into a two phase system composed of an opaque gel covered with an opalescent fluid on top. In contrast, there was little change in the solution clarity of the samples containing 0 to 1000:1 molar ratio of trehalose even after one months storage at 40° C. Although the 2–8° C. formulations were unchanged from the initial bmepoint, the sodium chloride containing samples were more opalescent.

The effect of trehalose on minimizing the soluble aggregate formation was assessed by SEC HPLC (FIG. **27**). No differences were observed between the trehalose formulations and the negative control which contained no tonicity modifier. The formulations appeared to degrade at the same rate to the same products upon storage at 40° C. The pH and concentration were also maintained over the duration of the study .

The presence of trehalose is beneficial in minimizing freeze-induced aggregation and is not deleterious to the stability in the liquid state. The candidate liquid multidose formulation preferably contains at least 500:1 molar ratio of trehalose to C2B8.

Stability of candidate liquid multidose formulation: Based on the aforementioned studies, a protoype liquid multidose formulation composed of 40 mg/mL rhuMAb CD20, 25 mM acetate, 150 mM trehalose, 0.9% benzyl alcohol and 0.02% polysorbate 20 at pH 5.0 was placed on stability at 2–8°, 30° C. and 40° C. The stability profile by SEC HPLC at each temperature studied is shown in FIG. **28**. Although the rate of aggregation is slightly faster at 40° C. for the multidose formulation (40 mg/mL) compared to the reference control (10 mg/mL rhuMAb CD20, 25 mM citrate, 150 mM sodium chloride, 0.07% polysorbate 80 at pH 6.5), no decrease in percentage monomer was observed upon storage at 2–8° C. for two years. The bioactivity of the two year old 2–8° C. samples was 99.2% relative to the reference control as determined by the CDC assay.

CONCLUSIONS

The above screening studies indicated that a stable high concentration rhuMAb CD20 liquid multidose formulation was possible by buffering with acetate, maintaining the pH at 5 and including preferably at least about 500 moles of trehalose per mole of antibody. The preferred liquid multidose configuration is composed of 40 mg/mL rhuMAb CD20, 25 mM acetate, 150 mM trehalose, 0.9% benzyl alcohol and 0.02% polysorbate 20 at pH 5 and has a shelf life of two years at 2–8° C.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 11

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 241 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

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

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Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
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Gly Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly Tyr Thr Phe Thr
            20                  25                  30

Glu Tyr Thr Met His Trp Met Arg Gln Ala Pro Gly Lys Gly Leu
            35                  40                  45

Glu Trp Val Ala Gly Ile Asn Pro Lys Asn Gly Gly Thr Ser His
            50                  55                  60

Asn Gln Arg Phe Met Asp Arg Phe Thr Ile Ser Val Asp Lys Ser
            65                  70                  75

Thr Ser Thr Ala Tyr Met Gln Met Asn Ser Leu Arg Ala Glu Asp
            80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Trp Arg Gly Leu Asn Tyr Gly
            95                 100                 105
```


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-continued

```
Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val
                110                 115                 120

Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
                125                 130                 135

Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly
                140                 145                 150

Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp
                155                 160                 165

Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
                170                 175                 180

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
                185                 190                 195

Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
                200                 205                 210

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys
                215                 220                 225

Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu
                230                 235                 240

Leu
241
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 214 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

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

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Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
  1                 5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Ile Asn
                 20                  25                  30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                 35                  40                  45

Leu Leu Ile Tyr Tyr Thr Ser Thr Leu His Ser Gly Val Pro Ser
                 50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile
                 65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                 80                  85                  90

Gly Asn Thr Leu Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu
                 95                 100                 105

Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
                110                 115                 120

Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu
                125                 130                 135

Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val
                140                 145                 150

Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu
                155                 160                 165

Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr
                170                 175                 180

Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu
                185                 190                 195
```


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-continued

```
Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn
                200                 205                 210

Arg Gly Glu Cys
            214
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

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

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Leu Gly Gly Arg Met Lys Gln Leu Glu Asp Lys Val Glu Glu Leu
  1               5                  10                  15

Leu Ser Lys Asn Tyr His Leu Glu Asn Glu Val Ala Arg Leu Lys
                20                  25                  30

Lys Leu Val Gly Glu Arg
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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

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

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Pro Lys Asn Ser Ser Met Ile Ser Asn Thr Pro
  1               5                  10  11
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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

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

```
His Gln Ser Leu Gly Thr Gln
  1               5       7
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

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

```
His Gln Asn Leu Ser Asp Gly Lys
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```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

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

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His Gln Asn Ile Ser Asp Gly Lys
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```

(2) INFORMATION FOR SEQ ID NO:8:

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-continued

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Val Ile Ser Ser His Leu Gly Gln
1 5 8

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2143 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GAATTCAACT TCTCCATACT TTGGATAAGG AAATACAGAC ATGAAAAATC 50

TCATTGCTGA GTTGTTATTT AAGCTTTGGA GATTATCGTC ACTGCAATGC 100

TTCGCAATAT GGCGCAAAAT GACCAACAGC GGTTGATTGA TCAGGTAGAG 150

GGGGCGCTGT ACGAGGTAAA GCCCGATGCC AGCATTCCTG ACGACGATAC 200

GGAGCTGCTG CGCGATTACG TAAAGAAGTT ATTGAAGCAT CCTCGTCAGT 250

AAAAAGTTAA TCTTTTCAAC AGCTGTCATA AAGTTGTCAC GGCCGAGACT 300

TATAGTCGCT TTGTTTTTAT TTTTTAATGT ATTTGTAACT AGAATTCGAG 350

CTCGCCGGGG ATCCTCTAGA GGTTGAGGTG ATTTTATGAA AAAGAATATC 400

GCATTTCTTC TTGCATCTAT GTTCGTTTTT TCTATTGCTA CAAACGCGTA 450

CGCTGATATC CAGATGACCC AGTCCCCGAG CTCCCTGTCC GCCTCTGTGG 500

GCGATAGGGT CACCATCACC TGTCGTGCCA GTCAGGACAT CAACAATTAT 550

CTGAACTGGT ATCAACAGAA ACCAGGAAAA GCTCCGAAAC TACTGATTTA 600

CTATACCTCC ACCCTCCACT CTGGAGTCCC TTCTCGCTTC TCTGGTTCTG 650

GTTCTGGGAC GGATTACACT CTGACCATCA GCAGTCTGCA ACCGGAGGAC 700

TTCGCAACTT ATTACTGTCA GCAAGGTAAT ACTCTGCCGC CGACGTTCGG 750

ACAGGGCACG AAGGTGGAGA TCAAACGAAC TGTGGCTGCA CCATCTGTCT 800

TCATCTTCCC GCCATCTGAT GAGCAGTTGA AATCTGGAAC TGCCTCTGTT 850

GTGTGCCTGC TGAATAACTT CTATCCCAGA GAGGCCAAAG TACAGTGGAA 900

GGTGGATAAC GCCCTCCAAT CGGGTAACTC CCAGGAGAGT GTCACAGAGC 950

AGGACAGCAA GGACAGCACC TACAGCCTCA GCAGCACCCT GACGCTGAGC 1000

AAAGCAGACT ACGAGAAACA CAAAGTCTAC GCCTGCGAAG TCACCCATCA 1050

GGGCCTGAGC TCGCCCGTCA CAAAGAGCTT CAACAGGGGA GAGTGTTAAG 1100

CTGATCCTCT ACGCCGGACG CATCGTGGCG CTAGTACGCA AGTTCACGTA 1150

AAAACGGTAT CTAGAGGTTG AGGTGATTTT ATGAAAAAGA ATATCGCATT 1200

TCTTCTTGCA TCTATGTTCG TTTTTTCTAT TGCTACAAAC GCGTACGCTG 1250

AGGTTCAGCT GGTGGAGTCT GGCGGTGGCC TGGTGCAGCC AGGGGGCTCA 1300

CTCCGTTTGT CCTGTGCAAC TTCTGGCTAC ACCTTTACCG AATACACTAT 1350

GCACTGGATG CGTCAGGCCC CGGGTAAGGG CCTGGAATGG GTTGCAGGGA 1400

TTAATCCTAA AAACGGTGGT ACCAGCCACA ACCAGAGGTT CATGGACCGT 1450

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```
TTCACTATAA GCGTAGATAA ATCCACCAGT ACAGCCTACA TGCAAATGAA          1500

CAGCCTGCGT GCTGAGGACA CTGCCGTCTA TTATTGTGCT AGATGGCGAG          1550

GCCTGAACTA CGGCTTTGAC GTCCGTTATT TTGACGTCTG GGGTCAAGGA          1600

ACCCTGGTCA CCGTCTCCTC GGCCTCCACC AAGGGCCCAT CGGTCTTCCC          1650

CCTGGCACCC TCCTCCAAGA GCACCTCTGG GGGCACAGCG GCCCTGGGCT          1700

GCCTGGTCAA GGACTACTTC CCCGAACCGG TGACGGTGTC GTGGAACTCA          1750

GGCGCCCTGA CCAGCGGCGT GCACACCTTC CCGGCTGTCC TACAGTCCTC          1800

AGGACTCTAC TCCCTCAGCA GCGTGGTGAC CGTGCCCTCC AGCAGCTTGG          1850

GCACCCAGAC CTACATCTGC AACGTGAATC ACAAGCCCAG CAACACCAAG          1900

GTCGACAAGA AAGTTGAGCC CAAATCTTGT GACAAAACTC ACACATGCCC          1950

GCCGTGCCCA GCACCAGAAC TGCTGGGCGG CCGCATGAAA CAGCTAGAGG          2000

ACAAGGTCGA AGAGCTACTC TCCAAGAACT ACCACCTAGA GAATGAAGTG          2050

GCAAGACTCA AAAAGCTTGT CGGGGAGCGC TAAGCATGCG ACGGCCCTAG          2100

AGTCCCTAAC GCTCGGTTGC CGCCGGGCGT TTTTTATTGT TAA                 2143
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(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 237 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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Met Lys Lys Asn Ile Ala Phe Leu Leu Ala Ser Met Phe Val Phe
-23         -20                 -15                 -10

Ser Ile Ala Thr Asn Ala Tyr Ala Asp Ile Gln Met Thr Gln Ser
            -5                   1                   5

Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr
        10                  15                  20

Cys Arg Ala Ser Gln Asp Ile Asn Asn Tyr Leu Asn Trp Tyr Gln
        25                  30                  35

Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Tyr Thr Ser
        40                  45                  50

Thr Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser
        55                  60                  65

Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp
        70                  75                  80

Phe Ala Thr Tyr Tyr Cys Gln Gln Gly Asn Thr Leu Pro Pro Thr
        85                  90                  95

Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala
        100                 105                 110

Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser
        115                 120                 125

Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg
        130                 135                 140

Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly
        145                 150                 155

Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr
        160                 165                 170

Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu
```


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```
        175                 180                 185

Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser
        190                 195                 200

Ser Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
        205                 210             214
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 300 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

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-23          -20                 -15                 -10

Ser Ile Ala Thr Asn Ala Tyr Ala Glu Val Gln Leu Val Glu Ser
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Gly Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Arg Leu Ser Cys
         10                  15                  20

Ala Thr Ser Gly Tyr Thr Phe Thr Glu Tyr Thr Met His Trp Met
         25                  30                  35

Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ala Gly Ile Asn
         40                  45                  50

Pro Lys Asn Gly Gly Thr Ser His Asn Gln Arg Phe Met Asp Arg
         55                  60                  65

Phe Thr Ile Ser Val Asp Lys Ser Thr Ser Thr Ala Tyr Met Gln
         70                  75                  80

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

Arg Trp Arg Gly Leu Asn Tyr Gly Phe Asp Val Arg Tyr Phe Asp
        100                 105                 110

Val Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr
        115                 120                 125

Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr
        130                 135                 140

Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe
        145                 150                 155

Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser
        160                 165                 170

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr
        175                 180                 185

Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr
        190                 195                 200

Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr Lys
        205                 210                 215

Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr
        220                 225                 230

Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Arg Met Lys
        235                 240                 245

Gln Leu Glu Asp Lys Val Glu Glu Leu Leu Ser Lys Asn Tyr His
        250                 255                 260

Leu Glu Asn Glu Val Ala Arg Leu Lys Lys Leu Val Gly Glu Arg
        265                 270                 275     277
```


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What is claimed is:

1. A stable aqueous pharmaceutical formulation comprising a therapeutically effective amount of an antibody not subjected to prior lyophilization, an acetate buffer from about pH 4.8 to about 5.5, a surfactant and a polyol, wherein the formulation lacks a tonicifying amount of sodium chloride.

2. The formulation of claim 1 which is isotonic.

3. The formulation of claim 1 which is stable at a temperature of about 2–8° C. for at least one year.

4. The formulation of claim 1 which is stable following freezing and thawing of the formulation.

5. The formulation of claim 1 which is stable at about 30° C. for at least one month.

6. The formulation of claim 1 wherein the polyol is a nonreducing sugar.

7. The formulation of claim 6 wherein the nonreducing sugar is trehalose.

8. The formulation of claim 6 wherein the nonreducing sugar is sucrose.

9. The formulation of claim 1 wherein the antibody is an antibody fragment.

10. The formulation of claim 9 wherein the antibody fragment is a $F(ab')_2$.

11. The formulation of claim 1 wherein the antibody binds CD18.

12. The formulation of claim 1 which is stable at a temperature of about 2–8° C. for at least two years.

13. The formulation of claim 1 wherein the antibody concentration in the formulation is from about 0.1 to about 50 mg/mL.

14. The formulation of claim 1 wherein the surfactant is a polysorbate.

15. The formulation of claim 1 wherein the antibody binds CD20.

16. The formulation of claim 15 wherein the acetate is present in an amount of about 5–30 mM.

17. The formulation of claim 16 wherein the acetate is present in an amount of 10–30 mM.

18. The formulation of claim 15 further comprising a preservative.

19. The formulation of claim 18 wherein the preservative is benzyl alcohol.

20. The formulation of claim 15 wherein the antibody is present in an amount of about 30–50 mg/mL.

21. The formulation of claim 20 wherein the buffer is 10–30 mM sodium acetate at pH 5, the polyol is trehalose in an amount of about 2–10% w/v, the surfactant is polysorbate in an amount of about 0.01–0.1% v/v, wherein the formulation further comprises benzyl alcohol as a preservative and wherein the formulation is stable at a temperature of about 2–8° C. for at least two years.

22. An article of manufacture comprising a container holding a stable aqueous pharmaceutical formulation comprising a therapeutically effective amount of an antibody not subjected to prior lyophilization, an acetate buffer from about pH 4.8 to about 5.5, a surfactant and a polyol, wherein the formulation lacks a tonicifying amount of sodium chloride.

23. A method for stabilizing an antibody in an aqueous pharmaceutical formulation by combining a therapeutically effective amount of an antibody not subjected to prior lyophilization, an acetate buffer from about pH 4.8 to about 5.5, a surfactant and a polyol, wherein the formulation lacks a tonicifying amount of sodium chloride.

24. A stable aqueous pharmaceutical formulation comprising a therapeutically effective amount of an antibody not subjected to prior lyophilization, a 10–30 mM acetate buffer at pH 5.0, a surfactant, and trehalose at a concentration of at least 134 mM, wherein the formulation lacks a tonicifying amount of sodium chloride.

25. The formulation of claim 24 wherein the antibody binds CD18 or CD20.

26. The formulation of claim 25 wherein the CD18 binding antibody is a $F(ab')_2$ fragment.

27. An article of manufacture comprising a container holding the formulation of claim 25.

28. The formulation of claim 1, wherein the acetate buffer is at pH 5.0.

29. The formulation of claim 28, wherein the antibody binds CD20.

* * * * *

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US 20050191298A1

(19) **United States**

(12) **Patent Application Publication** (10) **Pub. No.: US 2005/0191298 A1**

Bell et al. (43) **Pub. Date: Sep. 1, 2005**

(54) **METHOD OF TREATING HEMOLYTIC DISEASE**

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(21) Appl. No.: **11/050,543**

(22) Filed: **Feb. 3, 2005**

Related U.S. Application Data

(63) Continuation-in-part of application No. 10/771,552, filed on Feb. 3, 2004.

Publication Classification

(51) **Int. Cl.**[7] **A61K 48/00**; A61K 39/395

(52) **U.S. Cl.** **424/144.1**; 514/7; 514/44

(57) **ABSTRACT**

Paroxysmal nocturnal hemoglobinuria or other hemolytic diseases are treated using a compound which binds to or otherwise blocks the generation and/or the activity of one or more complement components, such as, for example, a complement-inhibiting antibody.

Exhibit no. D1
of the letter / report / expert opinion / plaint / defense
dated 09.08.2017
HOFFMANN - EITLE
Patent- und Rechtsanwälte
81925 München, Arabellastr. 30

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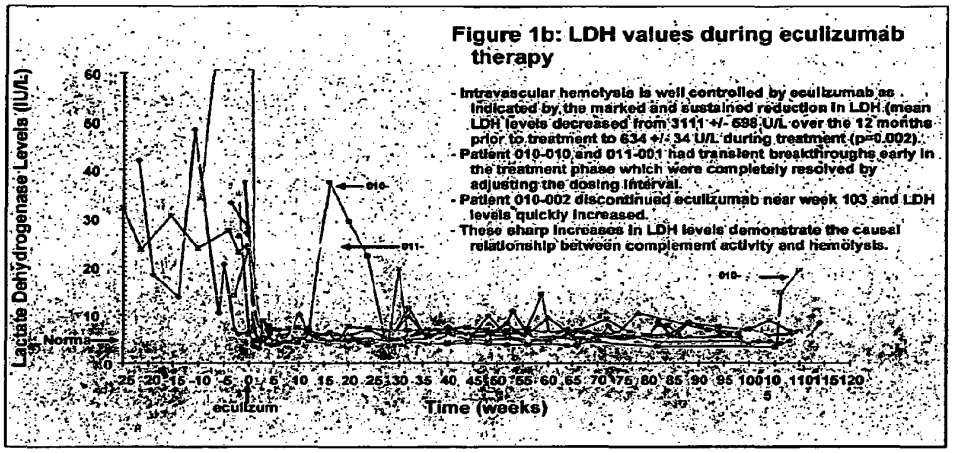
Figure 1A.
Biochemical Parameters of Hemolysis During Eculizumab Treatment

Biochemical Marker	Normal range	Time of Analysis			p-value[a]
		Pre-study[b]	12 weeks	64 weeks	
LDH (IU/L)	150 - 480	3110.7 +/- 598.4	594.0 +/- 31.7	622.4 +/-41.1	0.002
AST (IU/L)	10 - 40	76.2 +/- 16.0	26.2 +/- 2.3	30.1 +/- 3.2	0.02
Haptoglobin (g/L)	0.5 - 2	<0.06	<0.06	0.14 +/- 0.07[c]	N.S.[d]
Hemoglobin (g/dL)	11.5 - 18	10.0 +/- 0.4	10.3 +/- 0.4	10.4 +/- 0.4	N.S.
Bilirubin	3 - 15	25.9 +/- 4.3	28.2 +/- 4.4	28.7 +/- 4.0	N.S.
Reticulocytes ($x10^3/mm^3$)	20 - 80	161.4 +/- 25.9	191.2 +/- 23.6	189.6 +/- 21.8	N.S.

[a]from comparisons of mean change from pre-study to 64 weeks
[b]values represent means during 52 week period prior to treatment except for AST which represents the baseline mean
[c]10 of 11 patients were below the detectable limit of haptoglobin (<0.06 g/L); 1 patient had a value of 0.69 g/L.
[d]not significant

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Figure 1b



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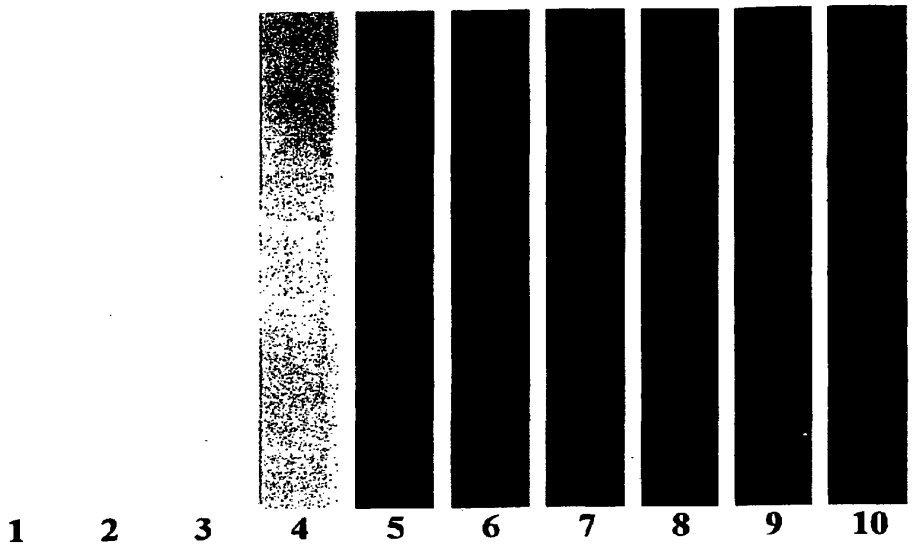
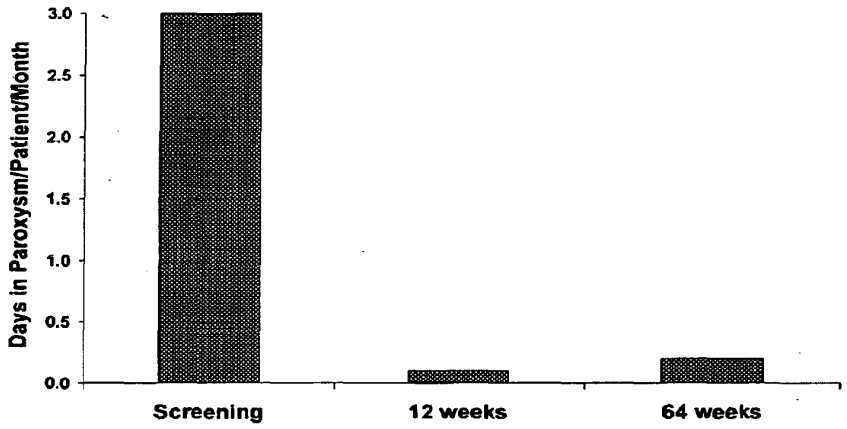


Figure 2

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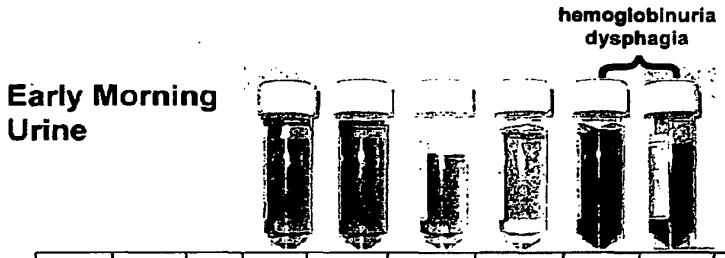
Figure 3.
Effect of Eculizumab on Paroxysm Rate (n=8)



Bars represent the paroxysm rates (number of paroxysms per patient per month) during the screening period (pre-eculizumab treatment), and during the first 12 weeks and the entire 64 weeks of eculizumab treatment. Three patients were not included in the analysis as either pretreatment urine scores were inadvertently not collected (2 patients) or an iron chelating agent that resulted in artificially colored urine was administered during the extension study (1 patient).

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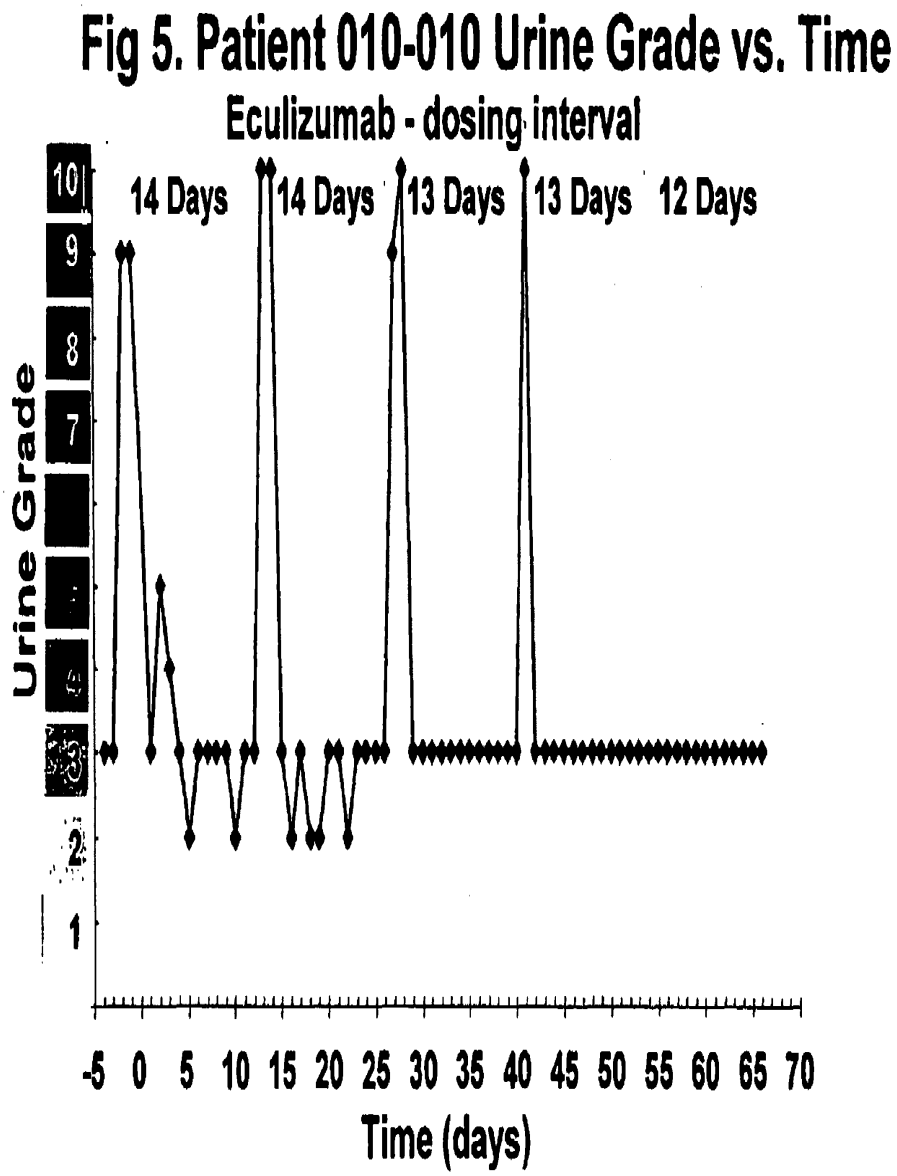
Figure 4.
Analysis of Complement Breakthrough



Visit	4							5			9
Day	0*	1-8	9	10	11	12	13	0*	1-2		0*
Urine	10	2-3	3	3	3	3	9	10	3		2
LDH	2624	–	784	–	–	697	1687	2917	–		495
AST	119	–	38	–	–	31	87	–	–		–
PK	28	–	51	–	–	35	31	23	–		51
PD	72	–	1	–	–	2	56	56	–		2

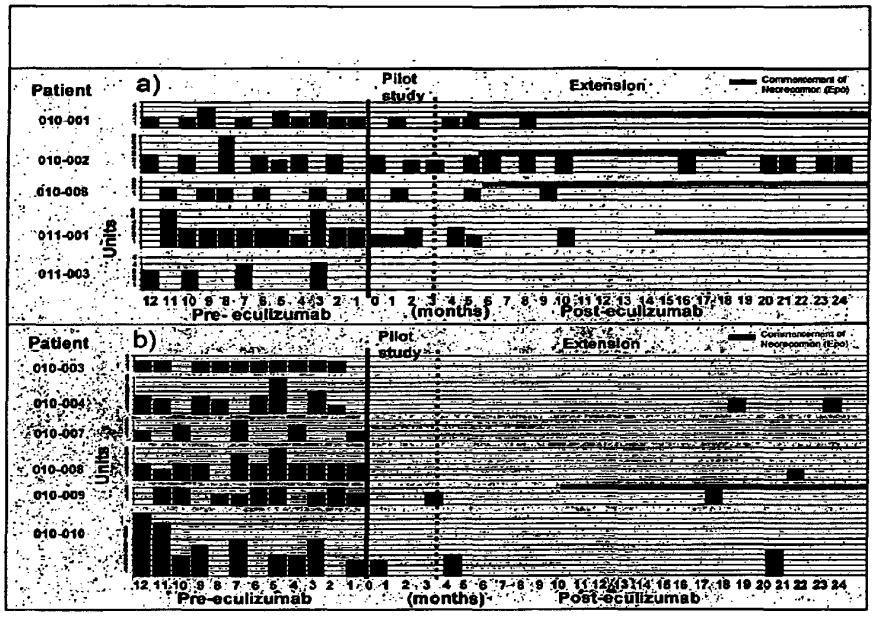
*Dose of eculizumab

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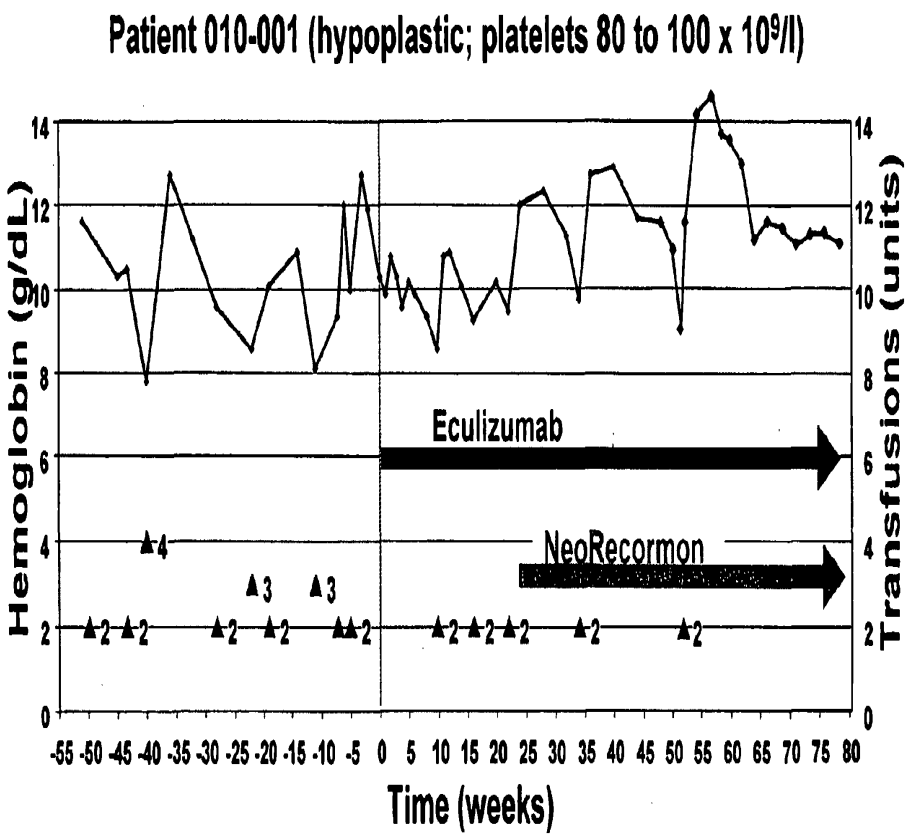
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Figure 6.
Units Transfused Pre- and Post-Eculizumab in (a) Cytopenic Patients and (b) Non-Cytopenic Patients



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Figure 7 Management of thrombocytopenic patient with erythropoietin



Transfusions reduced with eculizumab and transfusion independence with combined eculizumab and erythropoietin (NeoRecormon 18,000U 3x/week)

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Figure 8.
Eculizumab Pharmacodynamics Pre- and Post-dose

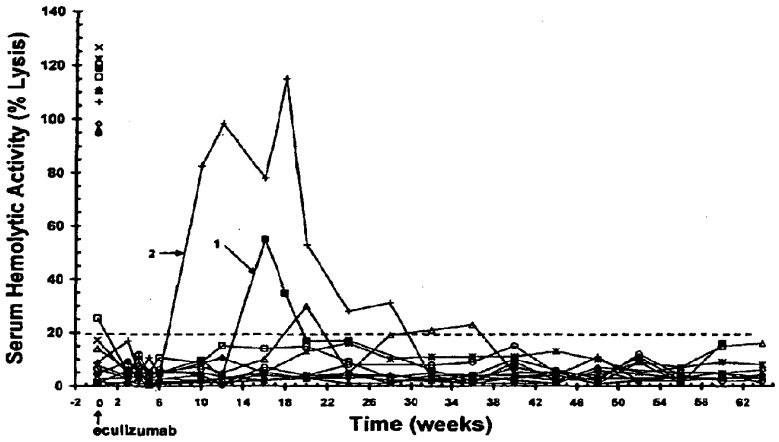


Figure 8 shows serum hemolytic activity (PD) during the 64 week treatment period as determined by the ability of serum to lyse antibody-presensitized chicken erythrocytes. The percentage of hemolytic activity at which complement is considered to be effectively inhibited (≤ 20 %) is indicated by dashed line. Two patients that demonstrated trough serum hemolytic activity values less than 20% are identified (patients 1 and 2).

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Figure 9.
Eculizumab Pharmacodynamics Pre- and Post-dose

Domain (a)	Mean Base-Line Score (b)	64 Week Change from Base-line (c)	p-value (d)
Global Health Status	56.1	13.8	0.009
Physical Functioning	70.9	14.3	<0.001
Emotional Functioning	70.5	12.5	<0.001
Role Functioning	66.7	14.5	0.003
Cognitive Functioning	77.3	10.3	0.001
Fatigue	47.5	-17.8	<0.001
Dyspnea	39.4	-16.6	0.002
Insomnia	30.3	-8.2	0.031
Pain	21.2	-8.2	0.023
Constipation	3.0	4.1	<0.001

a) the quality of life was assessed using the European Organization for Research and Treatment of Cancer QLQ-C30 instrument
b) numbers represent mean values of linearly transformed scores
c) values represent least-square means; positive change indicates improvement for Global Health Status and Functional Scales while a negative change indicates improvement for Symptom Scales
d) values are from a mixed analysis-of-covariance model with visit as a fixed effect, patient as a random effect, and baseline as a covariate

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Figure 10.
Symptoms Pre- and 2 Years Post-Eculizumab

Patient	Pre-Eculizumab			Post-Eculizumab		
	Abdominal Pain	Dysphagia	Erectile Dysfunction	Abdominal Pain	Dysphagia	Erectile Dysfunction
010-001	Every 4 – 8 weeks	Every 4 – 8 weeks	Every 4 – 8 weeks	None	None	None
010-002	None	None	--	None	None	--
010-003	Every week	Every week	Every week	None	None	None
010-004	Every 4 – 6 weeks	None	--	None	None	--
010-006	None	None	None	None	None	None
010-007	None	At least every 4 weeks	--	None	None	--
010-008	None	Every 10 weeks	Every 10 weeks	None	None	Intermittent
010-009	None	None	None	None	None	None
010-010	Every 4 weeks	Every 4 weeks	None	None	2 episodes in 2 years*	None
011-001	None	None	--	None	None	--
011-003	None	None	--	None	None	--

*This patient experienced a transient breakthrough in complement blockade with a return of hemolysis and symptoms. Increasing the dosing frequency to 12 days re-established complete complement blockade and prevented further symptoms.

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METHOD OF TREATING HEMOLYTIC DISEASE

RELATED APPLICATIONS

[0001] This application is a continuation-in-part of U.S. patent application Ser. No. 10/771,552, filed Feb. 3, 2004, the entire disclosure of which is incorporated herein by this reference.

BACKGROUND

[0002] 1. Technical Field

[0003] This disclosure relates to a method of treating a hemolytic disease such as, for example, paroxysmal nocturnal hemoglobinuria ("PNH"), by administering a compound which binds to, or otherwise blocks, the generation and/or activity of one or more complement components.

[0004] 2. Background of Related Art

[0005] Paroxysmal nocturnal hemoglobinuria ("PNH") is an uncommon blood disorder wherein red blood cells are compromised and are thus destroyed more rapidly than normal red blood cells. PNH results from a mutation of bone marrow cells resulting in the generation of abnormal blood cells. More specifically, PNH is believed to be a disorder of hematopoietic stem cells, which give rise to distinct populations of mature blood cells. The basis of the disease appears to be somatic mutations leading to the inability to synthesize the glycosyl-phosphatidylinositol ("GPI") anchor that is responsible for binding proteins to cell membranes. The mutated gene, PIG-A (phosphatidylinositol glycan class A) resides in the X chromosome and can have several different mutations, varying from deletions to point mutations.

[0006] PNH causes a sensitivity to complement proteins and this sensitivity occurs in the cell membrane. PNH cells are deficient in a number of proteins, particularly essential complement-regulating surface proteins. These complement-regulating surface proteins include the decay-accelerating factor ("DAF") or CD55 and membrane inhibitor of reactive lysis ("MIRL") or CD59.

[0007] PNH is characterized by hemolytic anemia (a decreased number of red blood cells), hemoglobinuria (the presence of hemoglobin in the urine particularly evident after sleeping), and hemoglobinemia (the presence of hemoglobin in the bloodstream). PNH-afflicted individuals are known to have paroxysms, which are defined here as incidences of dark-colored urine. Hemolytic anemia is due to intravascular destruction of red blood cells by complement components. Other known symptoms include dysphagia, fatigue, erectile dysfunction, thrombosis and recurrent abdominal pain.

[0008] Hemolysis resulting from hemolytic diseases causes local and systemic nitric oxide (NO) deficiency through the release of free hemoglobin. Free hemoglobin is a very efficient scavenger of NO, due in part to the accessibility of NO in the non-erythrocyte compartment and a 10^6 times greater affinity of the hememoiety for NO than that for oxygen. The occurrence of intravascular hemolysis often generates sufficient free hemoglobin to completely deplete haptoglobin. Once the capacity of this hemoglobin scavenging protein is exceeded, consumption of endogenous NO ensues. For example, in a setting of intravascular hemolysis such as PNH, where LDH levels can easily exceed 2-3 times their normal levels, free hemoglobin would likely obtain concentrations of 0.8-1.6 g/l. Since haptoglobin can only bind somewhere between 0.7 to 1.5 g/l of hemoglobin depending on the haptoglobin allotype, a large excess of free hemoglobin would be generated. Once the capacity of hemoglobin reabsorption by the kidney proximal tubules is exceeded, hemoglobinuria ensues. The release of free hemoglobin during intravascular hemolysis results in excessive consumption of NO with subsequent enhanced smooth muscle contraction, vasoconstriction and platelet activation and aggregation. PNH-related morbidities associated with NO scavenging by hemoglobin include abdominal pain, erectile dysfunction, esophageal spasm, and thrombosis.

[0009] The laboratory evaluation of hemolysis normally includes hematologic, serologic, and urine tests. Hematologic tests include an examination of the blood smear for morphologic abnormalities of RBCs (to determine causation), and the measurement of the reticulocyte count in whole blood (to determine bone marrow compensation for RBC loss). Serologic tests include lactate dehydrogenase (LDH; widely performed), and free hemoglobin (not widely performed) as a direct measure of hemolysis. LDH levels, in the absence of tissue damage in other organs, can be useful in the diagnosis and monitoring of patients with hemolysis. Other serologic tests include bilirubin or haptoglobin, as measures of breakdown products or scavenging reserve, respectively. Urine tests include bilirubin, hemosiderin, and free hemoglobin, and are generally used to measure gross severity of hemolysis and for differentiation of intravascular vs. extravascular etiologies of hemolysis rather than routine monitoring of hemolysis. Further, RBC numbers, RBC (i.e. cell-bound) hemoglobin, and hematocrit are generally performed to determine the extent of any accompanying anemia rather than as a measure of hemolytic activity per se.

[0010] Steroids have been employed as a therapy for hemolytic diseases and may be effective in suppressing hemolysis in some patients, although long term use of steroid therapy carries many negative side effects. Afflicted patients may require blood transfusions, which carry risks of infection. Anti-coagulation therapy may also be required to prevent blood clot formation. Bone marrow transplantation has been known to cure PNH, however, bone marrow matches are often very difficult to find and mortality rates are high with such procedure.

[0011] It would be advantageous to provide a treatment which safely and reliably eliminates and/or limits hemolytic diseases, such as PNH, and their effects.

SUMMARY

[0012] Paroxysmal nocturnal hemoglobinuria ("PNH") and other hemolytic diseases are treated in accordance with this disclosure using a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Suitable compounds include, for example, antibodies which bind to or otherwise block the generation and/or activity of one or more complement components, such as, for example, an antibody specific to complement component C5. In particularly useful embodiments, the compound is an anti-C5 antibody selected from the group consisting of h5G1.1-mAb (eculizumab), h5G1.1-scFv (pexelizumab) and other functional fragments of

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h5G1.1. It has surprisingly been found that the present methods provide improvements in the PNH subject within 24 hours of administration of the compound. For example, hemolysis is significantly reduced within 24 hours of administration of the compound as indicated by resolution of hemoglobinuria.

[0013] The complement-inhibiting compound can be administered prophylactically in individuals known to have a hemolytic disease to prevent, or help prevent the onset of symptoms. Alternatively, the complement-inhibiting compound can be administered as a therapeutic regimen to an individual experiencing symptoms of a hemolytic disease.

[0014] In another aspect, a method of increasing the proportion of complement sensitive type III red blood cells and therefore the total red blood cell count in a patient afflicted with a hemolytic disease is contemplated. The method comprises administering a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components to a patient afflicted with a hemolytic disease. By increasing type III red blood cell count, symptoms such as fatigue and anemia also can be alleviated in a patient afflicted with a hemolytic disease.

[0015] In yet another aspect, the present disclosure contemplates a method of rendering a subject afflicted with a hemolytic disease less dependent on transfusions or transfusion-independent by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components. It has surprisingly been found that patients can be rendered transfusion-independent in accordance with the present methods. Unexpectedly, transfusion-independence can be maintained in some embodiments for twelve months or more, long beyond the 120 day life cycle of red blood cells. In other embodiments, transfusion-independence can be maintained for two years or more. Treatment for six months or more is required for the evaluation of transfusion independence given the long half life of red blood cells.

[0016] In another aspect, the present disclosure contemplates a method of treating a nitric oxide (NO) imbalance in a subject by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream, thereby increasing serum levels of nitric oxide (NO). In particularly useful embodiments, NO homeostasis is restored wherein there is a resolution of symptoms attributable to NO deficiency.

[0017] In another aspect, the present disclosure contemplates a method of treating thrombosis in a subject by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

[0018] In another aspect, the present disclosure contemplates a method of treating fatigue in a subject afflicted with a hemolytic disease by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

[0019] In another aspect, the present disclosure contemplates a method of treating erectile dysfunction in a subject afflicted with a hemolytic disease by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

[0020] In another aspect, the present disclosure contemplates a method of treating abdominal pain in a subject afflicted with a hemolytic disease by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

[0021] In yet another aspect, the present disclosure contemplates a method of treating a subject afflicted with a hemolytic disease by administering: 1) one or more compounds known to increase hematopoiesis (for example, either by boosting production, eliminating stem cell destruction or eliminating stem cell inhibition) in combination with 2) a compound selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components. Suitable compounds known to increase hematopoiesis include, for example, steroids, immunosuppressants (such as, cyclosporin), anti-coagulants (such as, warfarin), folic acid, iron and the like, erythropoietin (EPO) and antithymocyte globulin (ATG), antilymphocyte globulin (ALG), EPO derivatives, and darbepoetin alfa (commercially available as Aranesp® from Amgen, Inc., Thousand Oaks, Calif. (Aranesp® is a man-made form of EPO produced in Chinese hamster ovary (CHO) cells by recombinant DNA technology)). In particularly useful embodiments, erythropoietin (EPO) (a compound known to increase hematopoiesis), EPO derivatives, or darbepoetin alfa may be administered in combination with an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1.

[0022] In yet another aspect, the present disclosure contemplates a method of treating one or more symptoms of hemolytic diseases in a subject where the proportion of type III red blood cells of the subject's total red blood cell content is greater than 10% before or during treatment, by administering a compound selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block

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the activity of one or more complement components, said compound being administered alone or in combination with one or more compounds known to increase hematopoiesis, such as EPO, EPO derivatives, or darbepoetin alfa.

[0023] In yet another aspect, the present disclosure contemplates a method of treating one or more symptoms of hemolytic diseases in a subject having a platelet count above 40,000 per microliter, by administering a compound selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components, said compound being administered alone or in combination with one or more compounds known to increase hematopoiesis, such as EPO, EPO derivatives, or darbepoetin alfa.

[0024] In yet another aspect, the present disclosure contemplates a method of treating one or more symptoms of a hemolytic diseases in a subject having a reticulocyte count above 80×10^9 per liter, by administering a compound selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components, said compound being administered alone or in combination with one or more compounds known to increase hematopoiesis, such as EPO, EPO derivatives, or darbepoetin alfa.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] **FIG. 1A** reports biochemical parameters of hemolysis measured during treatment of PNH patients with an anti-C5 antibody.

[0026] **FIG. 1B** graphically depicts the effect of treatment with an anti-C5 antibody on lactate dehydrogenase (LDH) levels.

[0027] **FIG. 2** shows a urine color scale devised to monitor the incidence of paroxysm of hemoglobinuria in PNH patients.

[0028] **FIG. 3** is a graph of the effects of eculizumab treatments on patient paroxysm rates, as compared to pre-treatment rates.

[0029] **FIG. 4** shows urine samples of PNH patients and measurements of hemoglobinuria, dysphagia, LDH, AST, pharmacokinetics (PK) and pharmacodynamics (PD) reflecting the immediate and positive effects of the present methods on hemolysis, symptoms and pharmacodynamics suitable to completely block complement.

[0030] **FIG. 5** graphically depicts the effect of anti-C5 antibody dosing schedule on hemoglobinuria over time.

[0031] **FIGS. 6*a*** and **6*b*** are graphs comparing the number of transfusion units required per patient per month, prior to and during treatment with an anti-C5 antibody: **FIG. 6*a*** depicts cytopenic patients; and **FIG. 6*b*** depicts non-cytopenic patients.

[0032] **FIG. 7** shows the management of a thrombocytopenic patient by administering an anti-C5 antibody and erythropoietin (EPO).

[0033] **FIG. 8** graphically depicts the pharmacodynamics of an anti-C5 antibody.

[0034] **FIG. 9** is a chart of the results of European Organization for Research and Treatment of Cancer questionnaires ("EORTC QLC-C30") completed during the anti-C5 therapy regimen addressing quality of life issues.

[0035] **FIG. 10** is a chart depicting the effects of anti-C5 antibody treatments on adverse symptoms associated with PNH.

DETAILED DESCRIPTION

[0036] The present disclosure relates to a method of treating paroxysmal nocturnal hemoglobinuria ("PNH") and other hemolytic diseases in mammals. Specifically, the methods of treating hemolytic diseases, which are described herein, involve using compounds which bind to or otherwise block the generation and/or activity of one or more complement components. The present methods have been found to provide surprising results. For instance, hemolysis rapidly ceases upon administration of the compound which binds to or otherwise blocks the generation and/or activity of one or more complement components, with hemoglobinuria being significantly reduced after treatment. Also, hemolytic patients can be rendered less dependent on transfusions or transfusion-independent for extended periods (twelve months or more), well beyond the 120 day life cycle of red blood cells. In addition, type III red blood cell count can be increased dramatically in the midst of other mechanisms of red blood cell lysis (non-complement mediated and/or earlier complement component mediated e.g., Cb3). Another example of a surprising result is that symptoms resolved, indicating that NO serum levels were increased enough even in the presence of other mechanisms of red blood cell lysis. These and other results reported herein are unexpected and could not be predicted from prior treatments of hemolytic diseases.

[0037] Any compound which binds to or otherwise blocks the generation and/or activity of one or more complement components can be used in the present methods. A specific class of such compounds which is particularly useful includes antibodies specific to a human complement component, especially anti-C5 antibodies. The anti-C5 antibody inhibits the complement cascade and, ultimately, prevents red blood cell ("RBC") lysis by the complement protein complex C5b-9. By inhibiting and/or reducing the lysis of RBCs, the effects of PNH and other hemolytic diseases (including symptoms such as hemoglobinuria, anemia, hemoglobinemia, dysphagia, fatigue, erectile dysfunction, recurrent abdominal pain and thrombosis) are eliminated or decreased.

[0038] In another embodiment, soluble forms of the proteins CD55 and CD59, singularly or in combination with each other, can be administered to a subject to inhibit the complement cascade in its alternative pathway. CD55 inhibits at the level of C3, thereby preventing the further progression of the cascade. CD59 inhibits the C5b-8 complex from combining with C9 to form the membrane attack complex (see discussion below).

[0039] The complement system acts in conjunction with other immunological systems of the body to defend against intrusion of bacterial and viral pathogens. There are at least

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25 proteins involved in the complement cascade, which are found as a complex collection of plasma proteins and membrane cofactors. Complement components achieve their immune defensive functions by interacting in a series of intricate but precise enzymatic cleavage and membrane binding events. The resulting complement cascade leads to the production of products with opsonic, immunoregulatory, and lytic functions. A concise summary of the biologic activities associated with complement activation is provided, for example, in The Merck Manual, 16th Edition.

[0040] The complement cascade progresses via the classical pathway, the alternative pathway or the lectin pathway. These pathways share many components, and while they differ in their initial steps, they converge and share the same "terminal complement" components (C5 through C9) responsible for the activation and destruction of target cells. The classical complement pathway is typically initiated by antibody recognition of and binding to an antigenic site on a target cell. The alternative pathway is usually antibody independent, and can be initiated by certain molecules on pathogen surfaces. Additionally, the lectin pathway is typically initiated with binding of mannose-binding lectin ("MBL") to high mannose substrates. These pathways converge at the point where complement component C3 is cleaved by an active protease to yield C3a and C3b.

[0041] C3a is an anaphylatoxin (see discussion below). C3b binds to bacteria and other cells, as well as to certain viruses and immune complexes, and tags them for removal from the circulation. (C3b in this role is known as opsonin.) The opsonic function of C3b is generally considered to be the most important anti-infective action of the complement system. Patients with genetic lesions that block C3b function are prone to infection by a broad variety of pathogenic organisms, while patients with lesions later in the complement cascade sequence, i.e., patients with lesions that block C5 functions, are found to be more prone only to Neisseria infection, and then only somewhat more prone (Fearon, in Intensive Review of Internal Medicine, 2nd Ed. Fanta and Minaker, eds. Brigham and Women's and Beth Israel Hospitals, 1983).

[0042] C3b also forms a complex with other components unique to each pathway to form classical or alternative C5 convertase, which cleaves C5 into C5a and C5b. C3 is thus regarded as the central protein in the complement reaction sequence since it is essential to all three activation pathways (Wurzner, et al., Complement Inflamm. 8:328-340, 1991). This property of C3b is regulated by the serum protease Factor I, which acts on C3b to produce iC3b (inactive C3b). While still functional as an opsonin, iC3b can not form an active C5 convertase.

[0043] The pro-C5 precursor is cleaved after amino acid 655 and 659, to yield the beta chain as an amino terminal fragment (amino acid residues +1 to 655 of the sequence) and the alpha chain as a carboxyl terminal fragment (amino acid residues 660 to 1658 of the sequence), with four amino acids (amino acid residues 656-659 of the sequence) deleted between the two. C5 is glycosylated, with about 1.5-3 percent of its mass attributed to carbohydrate. Mature C5 is a heterodimer of a 999 amino acid 115 kDa alpha chain that is disulfide linked to a 655 amino acid 75 kDa beta chain. C5 is found in normal serum at approximately 75 μg/ml (0.4 μM). C5 is synthesized as a single chain precursor protein product of a single copy gene (Haviland et al. J. Immunol. 1991, 146:362-368). The cDNA sequence of the transcript of this gene predicts a secreted pro-C5 precursor of 1658 amino acids along with an 18 amino acid leader sequence (see, U.S. Pat. No. 6,355,245).

[0044] Cleavage of C5 releases C5a, a potent anaphylatoxin and chemotactic factor, and leads to the formation of the lytic terminal complement complex, C5b-9. C5a is cleaved from the alpha chain of C5 by either alternative or classical C5 convertase as an amino terminal fragment comprising the first 74 amino acids of the alpha chain (i.e., amino acid residues 660-733 of the sequence). Approximately 20 percent of the 11 kDa mass of C5a is attributed to carbohydrate. The cleavage site for convertase action is at, or immediately adjacent to, amino acid residue 733 of the sequence. A compound that binds at, or adjacent, to this cleavage site would have the potential to block access of the C5 convertase enzymes to the cleavage site and thereby act as a complement inhibitor.

[0045] C5b combines with C6, C7, and C8 to form the C5b-8 complex at the surface of the target cell. Upon binding of several C9 molecules, the membrane attack complex ("MAC", C5b-9, terminal complement complex—TCC) is formed. When sufficient numbers of MACs insert into target cell membranes, the openings they create (MAC pores) mediate rapid osmotic lysis of the target cells. Lower, non-lytic concentrations of MACs can produce other proinflammatory effects. In particular, membrane insertion of small numbers of the C5b-9 complexes into endothelial cells and platelets can cause deleterious cell activation. In some cases activation may precede cell lysis.

[0046] C5a and C5b-9 also have pleiotropic cell activating properties, by amplifying the release of downstream inflammatory factors, such as hydrolytic enzymes, reactive oxygen species, arachidonic acid metabolites and various cytokines. C5 can also be activated by means other than C5 convertase activity. Limited trypsin digestion (Minta and Man, J. Immunol. 1977, 119:1597-1602; Wetsel and Kolb, J. Immunol. 1982, 128:2209-2216) and acid treatment (Yammamoto and Gewurz, J. Immunol. 1978, 120:2008; Damerau et al., Molec. Immunol. 1989, 26:1133-1142) can also cleave C5 and produce active C5b.

[0047] 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 proinflammatory granulocytes to the site of complement activation.

[0048] Any compounds which bind to or otherwise block the generation and/or activity of any of the human complement components may be utilized in accordance with the present disclosure. In some embodiments, antibodies specific to a human complement component are useful herein. Some compounds include antibodies directed against complement components C-1, C-2, C-3, C-4, C-5, C-6, C-7, C-8, C-9, Factor D, Factor B, Factor P, MBL, MASP-1, and MASP-2, thus preventing the generation of the anaphyla-

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toxic activity associated with C5a and/or preventing the assembly of the membrane attack complex associated with C5b.

[0049] Also useful in the present methods are naturally occurring or soluble forms of complement inhibitory compounds such as CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH. Other compounds which may be utilized to bind to or otherwise block the generation and/or activity of any of the human complement components include, but are not limited to, proteins, protein fragments, peptides, small molecules, RNA aptamers including ARC187 (which is commercially available from Archemix Corp., Cambridge, Mass.), L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, molecules which may be utilized in RNA interference (RNAi) such as double stranded RNA including small interfering RNA (siRNA), locked nucleic acid (LNA) inhibitors, peptide nucleic acid (PNA) inhibitors, etc.

[0050] Functionally, one suitable class of compounds inhibits the cleavage of C5, which blocks the generation of potent proinflammatory molecules C5a and C5b-9 (terminal complement complex). Preferably, the compound does not prevent the formation of C3b, which subserves critical immunoprotective functions of opsonization and immune complex clearance.

[0051] While preventing the generation of these membrane attack complex molecules, inhibition of the complement cascade at C5 preserves the ability to generate C3b, which is critical for opsonization of many pathogenic microorganisms, as well as for immune complex solubilization and clearance. Retaining the capacity to generate C3b appears to be particularly important as a therapeutic factor in complement inhibition for hemolytic diseases, where increased susceptibility to thrombosis, infection, fatigue, lethargy and impaired clearance of immune complexes are pre-existing clinical features of the disease process.

[0052] Particularly useful compounds for use herein are antibodies that reduce, directly or indirectly, the conversion of complement component C5 into complement components C5a and C5b. One class of useful antibodies are those having at least one antigen binding site and exhibiting specific binding to human complement component C5. Particularly useful complement inhibitors are compounds which reduce the generation of C5a and/or C5b-9 by greater than about 30%. Anti-C5 antibodies that have the desirable ability to block the generation of C5a have been known in the art since at least 1982 (Moongkarndi et al. Immunobiol. 1982, 162:397; Moongkarndi et al. Immunobiol. 1983, 165:323). Antibodies known in the art that are immunoreactive against C5 or C5 fragments include antibodies against the C5 beta chain (Moongkarndi et al. Immunobiol. 1982, 162:397; Moongkarndi et al. Immunobiol. 1983, 165:323; Wurzner et al. 1991, supra; Mollnes et al. Scand. J. Immunol. 1988, 28:307-312); C5a (see for example, Ames et al. J. Immunol. 1994, 152:4572-4581, U.S. Pat. No. 4,686,100, and European patent publication No. 0 411 306); and antibodies against non-human C5 (see for example, Giclas et al. J. Immunol. Meth. 1987, 105:201-209). Particularly useful anti-C5 antibodies are h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1. Methods for the preparation of h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1 are described in U.S. Pat. No. 6,355,245 and "Inhibition of Complement Activity by Humanized Anti-C5 Antibody and Single Chain Fv", Thomas et al., *Molecular Immunology*, Vol. 33, No. 17/18, pages 1389-1401, 1996, the disclosures of which are incorporated herein in their entirety by this reference. The antibody h5G1.1-mAb is currently undergoing clinical trials under the tradename eculizumab.

[0053] Hybridomas producing monoclonal antibodies reactive with complement component C5 can be obtained according to the teachings of Sims, et al., U.S. Pat. No. 5,135,916. Antibodies are prepared using purified components of the complement C5 component as immunogens according to known methods. In accordance with this disclosure, complement component C5, C5a or C5b is preferably used as the immunogen. In accordance with particularly preferred useful embodiments, the immunogen is the alpha chain of C5.

[0054] Particularly useful antibodies share the required functional properties discussed in the preceding paragraph and have any of the following characteristics:

[0055] (1) they compete for binding to portions of C5 that are specifically immunoreactive with 5G1.1;

[0056] (2) they specifically bind to the C5 alpha chain—such specific binding, and competition for binding can be determined by various methods well known in the art, including the plasmon surface resonance method (Johne et al., J. Immunol. Meth. 1993, 160:191-198); and

[0057] (3) they block the binding of C5 to either C3 or C4 (which are components of the C5 convertases).

[0058] The compound that inhibits the production and/or activity of at least one complement component can be administered in a variety of unit dosage forms. The dose will vary according to the particular compound employed. For example, different antibodies may have different masses and/or affinities, and thus require different dosage levels. Antibodies prepared as fragments (e.g., Fab, $F(ab')_2$, scFv) will also require differing dosages than the equivalent intact immunoglobulins, as they are of considerably smaller mass than intact immunoglobulins, and thus require lower dosages to reach the same molar levels in the patient's blood.

[0059] 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.

[0060] Administration of the compound that inhibits the production and/or activity of at least one complement component will generally be in an aerosol form with a suitable pharmaceutical carrier, via intravenous infusion by injection, subcutaneous injection, orally, or sublingually. Other routes of administration may be used if desired.

[0061] It is further contemplated that a combination therapy can be used wherein a complement-inhibiting compound is administered in combination with a regimen of known therapy for hemolytic disease. Such regimens include administration of 1) one or more compounds known to increase hematopoiesis (for example, either by boosting production, eliminating stem cell destruction or eliminating stem cell inhibition) in combination with 2) a compound

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selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components. Suitable compounds known to increase hematopoiesis include, for example, steroids, immunosuppressants (such as, cyclosporin), anticoagulants (such as, warfarin), folic acid, iron and the like, erythropoietin (EPO) and antithymocyte globulin (ATG), antilymphocyte globulin (ALG), EPO derivatives, and darbepoetin alfa (commercially available as Aranesp® from Amgen, Inc., Thousand Oaks, Calif. (Aranesp® is a man-made form of EPO produced in Chinese hamster ovary (CHO) cells by recombinant DNA technology)). In particularly useful embodiments, erythropoietin (EPO) (a compound known to increase hematopoiesis), EPO derivatives, or darbepoetin alfa may be administered in combination with an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1.

[0062] 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.

[0063] The present disclosure contemplates methods of reducing hemolysis in a patient afflicted with a hemolytic disease by administering one or more compounds which bind to or otherwise block the generation and/or activity of one or more complement components. Reducing hemolysis means that the duration of time a person suffers from hemolysis is reduced by about 25% or more. The effectiveness of the treatment can be evaluated in any of the various manners known to those skilled in the art for determining the level of hemolysis in a patient. One qualitative method for detecting hemolysis is to observe the occurrences of hemoglobinuria. Quite surprisingly, treatment in accordance with the present methods reduces hemolysis as determined by a rapid reduction in hemoglobinuria.

[0064] A more qualitative manner of measuring hemolysis is to measure lactate dehydrogenase (LDH) levels in the patient's bloodstream. LDH catalyzes the interconversion of pyruvate and lactate. Red blood cells metabolize glucose to lactate, which is released into the blood and is taken up by the liver. LDH levels are used as an objective indicator of hemolysis. As those skilled in the art will appreciate, measurements of "upper limit of normal" levels of LDH will vary from lab to lab depending on a number of factors including the particular assay employed and the precise manner in which the assay is conducted. Generally speaking, however, the present methods can reduce hemolysis in a patient afflicted with a hemolytic disease as reflected by a reduction of LDH levels in the patients to within 20% of the upper limit of normal LDH levels. Alternatively, the present methods can reduce hemolysis in a patient afflicted with a hemolytic disease as reflected by a reduction of LDH levels in the patients of greater than 50% of the patient's pre-treatment LDH level, preferably greater than 65% of the patient's pre-treatment LDH level, most preferably greater than 80% of the patient's pre-treatment LDH level.

[0065] Another quantitative measurement of a reduction in hemolysis is the presence of GPI-deficient red blood cells (Type III red blood cells). As those skilled in the art will appreciate, Type III red blood cells have no GPI-anchor protein expression on the cell surface. The proportion of GPI-deficient cells can be determined by flow cytometry using, for example, the technique described in Richards, et al., *Clin. Appl. Immunol. Rev., vol.* 1, pages 315-330, 2001. The present methods can reduce hemolysis in a patient afflicted with a hemolytic disease as reflected by an increase in Type III red blood cells. Preferably an increase in Type III red blood cell levels in the patient of greater than 25% of the total red blood cell count is achieved, more preferably an increase in Type III red blood cell levels in the patients greater than 50% of the total red blood cell count is achieved, most preferably an increase in Type III red blood cell levels in the patients greater than 75% of the total red blood cell count is achieved.

[0066] Methods of reducing one or more symptoms associated with PNH or other hemolytic diseases are also within the scope of the present disclosure. Such symptoms include, for example, abdominal pain, fatigue, dyspnea and insomnia. Symptoms can be the direct result of lysis of red blood cells (e.g., hemoglobinuria, anemia, fatigue, low red blood cell count, etc.) or the symptoms can result from low nitric oxide (NO) levels in the patient's bloodstream (e.g., abdominal pain, erectile dysfunction, dysphagia, thrombosis, etc.). It has recently been reported that almost all patients with greater than 40% PNH type III granulocyte clone have thrombosis, abdominal pain, erectile dysfunction and dysphagia, indicating a high hemolytic rate (see Moyo et al., British J. Haematol. 126:133-138 (2004)).

[0067] In particularly useful embodiments, the present methods provide a reduction in one or more symptoms associated with PNH or other hemolytic diseases in a patient having a platelet count in excess of 40,000 per microliter (a hypoplastic patient), preferably in excess of 75,000 per microliter, most preferably in excess of 150,000 per microliter. In other embodiments, the present methods provide a reduction in one or more symptoms associated with PNH or other hemolytic diseases in a patient where the proportion of PNH type III red blood cells of the subject's total red blood cell content is greater than 10%, preferably greater than 25%, most preferably in excess of 50%. In yet other embodiments, the present methods provide a reduction in one or more symptoms associated with PNH or other hemolytic diseases in a patient having a reticulocyte count in excess of 80×10^9 per liter, more preferably in excess of 120×10^9 per liter, most preferably in excess of 150×10^9 per liter. Patients in the most preferable ranges recited above have active bone marrow and will produce adequate numbers of red blood cells. While in a patient afflicted with PNH or other hemolytic disease the red blood cells may be defective in one or more ways (e.g., GPI deficient), the present methods are particularly useful in protecting such cells from lysis resulting from complement activation. Thus, patients within the preferred ranges benefit most from the present methods.

[0068] In one aspect, a method of reducing fatigue is contemplated, the method including the step of administer-

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ing to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing fatigue means the duration of time a person suffers from fatigue is reduced by about 25% or more. Fatigue is a symptom believed to be associated with intravascular hemolysis as the fatigue relents when hemoglobinuria resolves even in the presence of anemia. By reducing the lysis of red blood cells, the present methods reduce fatigue. Patients within the above-mentioned preferred ranges of type III red blood cells, reticulocytes and platelets benefit most from the present methods.

[0069] In another aspect, a method of reducing abdominal pain is contemplated, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing abdominal pain means the duration of time a person suffers from abdominal pain is reduced by about 25% or more. Abdominal pain is a symptom resulting from the inability of a patient's natural levels of haptoglobin to process all the free hemoglobin released into the bloodstream as a result of intravascular hemolysis, resulting in the scavenging of NO and intestinal dystonia and spasms. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream, thereby reducing abdominal pain. Patients within the above-mentioned preferred ranges of type III red blood cells, reticulocytes and platelets benefit most from the present methods.

[0070] In another aspect, a method of reducing dysphagia is contemplated, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing dysphagia means the duration of time a person has dysphagia attacks is reduced by about 25% or more. Dysphagia is a symptom resulting from the inability of a patient's natural levels of haptoglobin to process all the free hemoglobin released into the bloodstream as a result of intravascular hemolysis, resulting in the scavenging of NO and esophageal spasms. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream, thereby reducing dysphagia. Patients within the above-mentioned preferred ranges of type III red blood cells, reticulocytes and platelets benefit most from the present methods.

[0071] In yet another aspect, a method of reducing erectile dysfunction is contemplated, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing erectile dysfunction means the duration of time a person suffers from erectile dysfunction is reduced by about 25% or more. Erectile dysfunction is a symptom believed to be associated with scavenging of NO by free hemoglobin released into the bloodstream as a result of intravascular hemolysis. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream, thereby increasing serum levels of NO and reducing erectile dysfunction. Patients within the above-mentioned preferred ranges of type III red blood cells, reticulocytes and platelets benefit most from the present methods.

[0072] In yet another aspect, a method of reducing hemoglobinuria is contemplated, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing hemoglobinuria means a reduction in the number of times a person has red, brown, or darker urine, wherein the reduction is typically about 25% or more. Hemoglobinuria is a symptom resulting from the inability of a patient's natural levels of haptoglobin to process all the free hemoglobin released into the bloodstream as a result of intravascular hemolysis. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream and urine thereby reducing hemoglobinuria. Quite surprisingly, the reduction in hemoglobinuria occurs rapidly. Patients within the above-mentioned preferred ranges of type III red blood cells, reticulocytes and platelets benefit most from the present methods.

[0073] In still another aspect, a method of reducing thrombosis is contemplated, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing thrombosis means the duration of time a person has thrombosis attacks is reduced by about 25% or more. Thrombosis is a symptom believed to be associated with scavenging of NO by free hemoglobin released into the bloodstream as a result of intravascular hemolysis and/or the lack of CD59 on the surface of platelets resulting in terminal complement mediated activation of the platelet. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream, thereby increasing serum levels of NO and reducing thrombosis. In addition, blockade of complement will prevent terminal complement-mediated activation of platelets and thrombosis. C5a will also be inhibited by this method which can induce platelet aggregation through C5a receptors on platelets and endothelial cells.

[0074] Thrombosis is thought to be multi-factorial in etiology including NO scavenging by free hemoglobin, the absence of terminal complement inhibition on the surface of circulating platelets and changes in the endothelium surface by cell free heme. The intravascular release of free hemoglobin may directly contribute to small vessel thrombosis. NO has been shown to inhibit platelet aggregation, induce disaggregation of aggregated platelets and inhibit platelet adhesion. Conversely, NO scavenging by hemoglobin or the reduction of NO generation by the inhibition of arginine metabolism results in an increase in platelet aggregation. PNH platelets also lack the terminal complement inhibitor CD59 and multiple studies have shown that deposition of terminal complement (C5b-9) on platelets causes membrane vesiculation and the generation of microvesicles. The microvesicles act as a site for the generation of the clotting components factor Va, Xa or the prothrombinase complex. It is thought that these particles may also contribute to the genesis of thrombosis in PNH. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream, thereby increasing serum

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levels of NO and reducing thrombosis. In addition, inhibiting complement at C5 will prevent C5b-9 and C5a mediated activation of platelets and/or endothelial cells.

[0075] In particularly useful embodiments, the present methods reduce thrombosis, especially patients having a platelet count in excess of 40,000 per microliter, preferably in excess of 75,000 per microliter, most preferably in excess of 150,000 per microliter. In other embodiments, the present methods reduce thrombosis in patients where the proportion of PNH type III red blood cells of the subject's total red blood cell content is greater than 10%, preferably greater than 25%, more preferably in excess of 50%, most preferably in excess of 75%. In yet other embodiments, the present methods reduce transfusion thrombosis in patients having a reticulocyte count in excess of 80×10^9 per liter, more preferably in excess of 120×10^9 per liter, most preferably in excess of 150×10^9 per liter.

[0076] In still another aspect, a method of reducing anemia is contemplated, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing anemia means the duration of time a person has anemia is reduced by about 25% or more. Anemia in hemolytic diseases results from the blood's reduced capacity to carry oxygen due to the loss of red blood cell mass. By reducing the lysis of red blood cells, the present methods assist red blood cell levels to increase thereby reducing anemia.

[0077] In another aspect, a method of increasing the proportion of complement sensitive type III red blood cells and therefore the total red blood cell count in a patient afflicted with a hemolytic disease is contemplated. By increasing the patient's RBC count, fatigue, anemia and the patient's need for blood transfusions is reduced. The reduction in transfusions can be in frequency of transfusions, amount of blood units transfused, or both.

[0078] The method of increasing red blood cell count in a patient afflicted with a hemolytic disease includes the step of administering a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components to a patient afflicted with a hemolytic disease. In particularly useful embodiments, the present methods increase red blood cell count in a patient afflicted with a hemolytic disease, especially patients having a platelet count in excess of 40,000 per microliter, preferably in excess of 75,000 per microliter, most preferably in excess of 150,000 per microliter. In other embodiments, the present methods increase red blood cell count in a patient afflicted with a hemolytic disease where the proportion of PNH type III red blood cells of the subject's total red blood cell content is greater than 10%, preferably greater than 25%, more preferably in excess of 50%, most preferably in excess of 75%. In yet other embodiments, the present methods increase red blood cell count in a patient afflicted with a hemolytic disease having a reticulocyte count in excess of 80×10^9 per liter, more preferably in excess of 120×10^9 per liter, most preferably in excess of 150×10^9 per liter. In some embodiments, the methods of the present disclosure may result in a decrease in the frequency of transfusions by about 50%, typically a decrease in the frequency of transfusions by about 70%, more typically a decrease in the frequency of transfusions by about 90%.

[0079] In yet another aspect, the present disclosure contemplates a method of rendering a subject afflicted with a hemolytic disease less dependent on transfusions or transfusion-independent by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components. As those skilled in the art will appreciate, the normal life cycle for a red blood cell is about 120 days. Treatment for six months or more is required for the evaluation of transfusion independence given the long half life of red blood cells. It has unexpectedly been found that in some patients transfusion-independence can be maintained for twelve months or more, in some cases more than two years, long beyond the 120 day life cycle of red blood cells. In particularly useful embodiments, the present methods provide decreased dependence on transfusions or transfusion-independence in a patient afflicted with a hemolytic disease, especially patients having a platelet count in excess of 40,000 per microliter, preferably in excess of 75,000 per microliter, most preferably in excess of 150,000 per microliter. In other embodiments, the present methods provide decreased dependence on transfusions or transfusion-independence in a patient afflicted with a hemolytic disease where the proportion of PNH type III red blood cells of the subject's total red blood cell content is greater than 10%, preferably greater than 25%, more preferably in excess of 50%, most preferably in excess of 75%. In yet other embodiments, the present methods provide decreased dependence on transfusions or transfusion-independence in a patient afflicted with a hemolytic disease having a reticulocyte count in excess of 80×10^9 per liter, more preferably in excess of 120×10^9 per liter, most preferably in excess of 150×10^9 per liter.

[0080] Methods of increasing the nitric oxide (NO) levels in a patient having PNH or some other hemolytic disease are also within the scope of the present disclosure. These methods of increasing NO levels include the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Low NO levels arise in patients afflicted with PNH or other hemolytic diseases as a result of scavenging of NO by free hemoglobin released into the bloodstream as a result of intravascular hemolysis. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream, thereby increasing serum levels of NO. In particularly useful embodiments, NO homeostasis is restored as evidenced by a resolution of symptoms attributable to NO deficiencies.

EXAMPLES

[0081] Eleven patients participated in therapy trials to evaluate the effects of anti-C5 antibody on PNH and symptoms associated therewith. PNH patients were transfusion-dependent and hemolytic. Patients were defined as transfusion dependent with a history of four or more transfusions within twelve months. The median number of transfusions within the patient pool was nine in the previous twelve months. The median number of transfusion units used in the previous twelve months was twenty-two for the patient pool.

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[0082] Over the course of four weeks, each of 11 patients received a weekly 600 mg intravenous infusion of anti-C5 antibody for approximately thirty minutes. The specific anti-C5 antibody used in the study was eculizumab. Patients received 900 mg of eculizumab 1 week later then 900 mg on a bi-weekly basis. The first twelve weeks of the study constituted the pilot study. Following completion of the initial acute phase twelve week study, all patients participated in an extension study conducted to a total of 64 weeks. Ten of the eleven patients participated in an extension study conducted to a total of two years.

[0083] The effect of anti-C5 antibody treatments on PNH type III red blood cells ("RBCs") was tested. "PNH Type" refers to the density of GPI-anchored proteins expressed on the cell surface. Type I is normal expression, Type II is intermediate expression, and Type III has no GPI-anchor protein expression on the cell surface. The proportion of GPI-deficient cells is determined by flow cytometry in the manner described in Richards, et al., *Clin. Appl. Immunol. Rev,* vol.1, pages 315-330, 2001. As compared to pre-therapy conditions, PNH Type III red blood cells increased more than 50% during the extension study. The increase from a pre-study mean value of 36.7% of all red blood cells to a 64 week mean value of 58.4% of Type III red blood cells indicated that hemolysis had decreased sharply. See Table 1, below. Eculizumab therapy protected PNH type III RBCs from complement-mediated lysis, prolonging the cells survival. This protection of the PNH-affected cells reduced the need for transfusions, paroxysms and overall hemolysis in all patients in the trial.

TABLE 1

PNH Cell Populations Pre- and Post-Eculizumab Treatment in All Patients

PNH Cell Type	Proportion of PNH Cells (%) baseline	12 weeks	64 weeks	p-value[a]
Type III RBCs	36.7 +/- 5.9	59.2 +/- 8.0	58.4 +/- 8.5	0.005
Type II RBCs	5.3 +/- 1.4	7.5 +/- 2.1	13.2 +/- 2.4	0.013
Type III WBCs	92.1 +/- 4.6	89.9 +/- 6.6	91.1 +/- 5.8	N.S.
Type III Platelets	92.4 +/- 2.4	93.3 +/- 2.8	92.8 +/- 2.6	N.S.

[a]comparison of mean change from baseline to 64 weeks

[0084] During the course of the two year extension study, it was found that PNH red cells with a complete deficiency of GPI-linked proteins (Type III red cells) progressively increased during the treatment period from a mean of 36.7% to 58.9% (p=0.001) while partially deficient PNH red cells (Type II) increased from 5.3% to 8.7% (p=0.01). There was no concomitant change in the proportion of PNH neutrophils in any of the patients during eculizumab therapy, indicating that the increase in the proportion of PNH red cells was due to a reduction in hemolysis and transfusions rather than a change in the PNH clone(s) themselves.

[0085] The effect of anti-C5 antibody treatments on lactate dehydrogenase levels ("LDH") was measured on all eleven patients. LDH catalyzes the interconversion of pyruvate and lactate. Red blood cells metabolize glucose to lactate, which is released into the blood and is taken up by the liver. LDH levels are used as an objective indicator of hemolysis. The LDH levels were decreased by greater than 80% as compared to pre-treatment levels. The LDH levels were lowered from a pre-study mean value of 3111 U/L to a mean value of 594 U/L during the pilot study and a mean value of 622 U/L after 64 weeks (p=0.002 for 64 week comparison; see **FIGS. 1A and 1B**).

[0086] Similarly, aspartate aminotransferase (AST) levels, another marker of red blood cell hemolysis, decreased from a mean baseline value of 76 IU/L to 26 IU/L and 30 IU/L during the 12 and 64 weeks of treatment, respectively (p=0.02 for 64 week comparison). Levels of haptoglobin, hemoglobin and bilirubin, and numbers of reticulocytes, did not change significantly from prestudy values during the 64 week treatment period.

[0087] Paroxysm rates were measured and compared to pre-treatment levels. Paroxysm as used in this disclosure is defined as incidences of dark-colored urine with a colorimetric level of 6 of more on a scale of 1-10. **FIG. 2** shows the urine color scale devised to monitor the incidence of paroxysm of hemoglobinuria in patients with PNH before and during treatment. As compared to pre-treatment levels, the paroxysm percentage rate was reduced by 93% (see, **FIG. 3**) from 3.0 paroxysms per patient per month before eculizumab treatment to 0.1 paroxysms per patient per month during the initial 12 weeks and 0.2 paroxysms per patient per month during the 64 week treatment (**FIG. 3** (p<0.001)).

[0088] 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.

[0089] The patients' need for transfusions was also reduced by the treatment with eculizumab. **FIG. 6*a*** compares the number of transfusion units required per patient per month, prior to and during treatment with an anti-C5 antibody for cytopenic patients, while **FIG. 6b** compares the number of transfusion units required per patient per month, prior to and during treatment with an anti-C5 antibody for non-cytopenic patients. A significant reduction in the need for transfusion was also noted in the entire group (mean transfusion rates decreased from 2.1 units per patient per month during a 1 year period prior to treatment to 0.6 units

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per patient per month during the initial 12 weeks and 0.5 units per patient per month during the combined 64 week treatment period), with non-cytopenic patients benefiting the most. In fact, four of the non-thrombocytopenic patients with normal platelet counts (≧150,000 per microliter) became transfusion-independent during the 64 week treatment.

[0090] The effect of eculizumab administered in combination with erythropoietin (EPO) was also evaluated in a thrombocytopenic patient. EPO (NeoRecormon®, Roche Pharmaceuticals, Basel, Switzerland) was administered in an amount of 18,000 I.U. three times per week beginning in week 23 of the study. As shown in **FIG. 7**, the frequency of transfusions required for this patient was significantly reduced, and soon halted.

[0091] For the two year extension study, 10 of the 11 patients from the initial 3 month study continued to receive 900 mg of eculizumab every other week. (One patient discontinued eculizumab therapy after 23 months.) Six of the 11 patients had normal platelets (no clinical evidence of marrow failure) whereas 5 of the 11 had low platelets. For the patient who discontinued eculizumab therapy after 23 months, intravascular hemolysis was successfully controlled by eculizumab, but the patient continued to be transfused even after erythropoietin therapy. This patient had the most severe hypoplasia at the start of eculizumab therapy with a platelet count below 30×10^9/l, suggesting that the ongoing transfusions were likely a result of the underlying bone marrow failure.

[0092] Results of the two year extension study also demonstrated that there was a statistically significant decrease in transfusion requirements for the patients. Three patients remained transfusion independent during the entire two year treatment period, and four cytopenic patients became transfusion independent, three following treatment with EPO (NeoRecormon®). The reduction in transfusion requirements was found to be most pronounced in patients with a good marrow reserve.

[0093] Pharmacodynamic levels were measured and recorded according to eculizumab doses. The pharmacodynamic analysis of eculizumab was determined by measuring the capacity of patient serum samples to lyse chicken erythrocytes in a standard total human serum complement hemolytic assay. Briefly, patient samples or human control serum (Quidel, San Diego Calif.) was diluted to 40% vol/vol with gelatin veronal-buffered saline (GVB2+, Advanced Research technologies, San Diego, Calif.) and added in triplicate to a 96-well plate such that the final concentrations of serum in each well was 20%. The plate was then incubated at room temperature while chicken erythrocytes (Lampire Biologics, Malvern, Pa.) were washed. The chicken erythrocytes were sensitized by the addition of anti-chicken red blood cell polyclonal antibody (0.1% vol/vol). The cells were then washed and resuspended in GVB2+ buffer. Chicken erythrocytes (2.5×10^6 cells/30 μL) were added to the plate containing human control serum or patient samples and incubated at 37° C. for 30 min. Each plate contained six additional wells of identically prepared chicken erythrocytes of which four wells were incubated with 20% serum containing 2 mM EDTA as the blank and two wells were incubated with GVB2+ buffer alone as a negative control for spontaneous hemolysis. The plate was then centrifuged and the supernatant transferred to a new flat bottom 96-well plate. Hemoglobin release was determined at OD 415 nm using a microplate reader. The percent hemolysis was determined using the following formula:

$$\text{Percent Hemolysis} = 100\times\frac{(OD\text{ patient sample} - OD\text{ blank})}{(OD\text{ human serum control} - OD\text{ blank})}$$

[0094] The graph of the pharmacodynamics (**FIG. 8**), the study of the physiological effects, shows the percentage of serum hemolytic activity (i.e. the percentage of cell lysis) over time. Cell lysis was dramatically reduced in the majority of the patients to below 20% of normal serum complement activity while under eculizumab treatment. Two patients exhibited a breakthrough in complement activity, but complement blockade was permanently restored by reducing the dosing interval to 12 days (See, **FIG. 4**).

[0095] Improvement of quality of life issues was also evaluated using the European Organization for Research and Treatment of Cancer Core (http://www.eortc.be) questionnaires ("EORTC QLC-C30"). Each of the participating patients completed the QLC-30 questionnaire before and during the eculizumab therapy. Overall improvements were observed in global health status, physical functioning, role functioning, emotional functioning, cognitive functioning, fatigue, pain, dyspnea and insomnia. (See **FIG. 9**).

[0096] Patients in the two year study experienced a reduction in adverse symptoms associated with PNH. For example, as set forth in **FIG. 10**, there was a demonstrated decrease of abdominal pain, dysphagia, and erectile dysfunction after administration of eculizumab in those patients reporting those symptoms before administration of eculizumab.

[0097] 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.

What is claimed is:

1. A method of rendering a subject afflicted with a hemolytic disease transfusion independent comprising administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components, wherein the subject is transfusion independent for over two years from the first administration of said compound.

2. A method as in claim 1 wherein the step of administering comprises administering an anti-C5 antibody.

3. A method as in claim 1 wherein the step of administering comprises administering an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.

4. A method as in claim 1 wherein the step of administering comprises administering a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibi-

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tors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

5. A method as in claim 1 wherein the step of administering comprises administering a compound selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

6. A method as in claim 1 further comprising the step of administering one or more compounds that increase hematopoiesis in combination with said compound.

7. A method as in claim 6 wherein the one or more compounds that increase hematopoiesis is selected from the group consisting of steroids, immunosuppressants, anticoagulants, folic acid, iron, erythropoietin (EPO), antithymocyte globulin (ATG) and antilymphocyte globulin (ALG).

8. A method as in claim 1 wherein EPO is administered in combination with an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.

9. A method as in claim 1 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 10%.

10. A method as in claim 1 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 25%.

11. A method as in claim 1 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 50%.

12. A method as in claim 1 wherein the subject's platelet count is greater than 40,000 per microliter.

13. A method as in claim 1 wherein the subject's platelet count is greater than 75,000 per microliter.

14. A method as in claim 1 wherein the subject's platelet count is greater than 150,000 per microliter.

15. A method as in claim 1 wherein the subject's reticulocyte count is greater than 80×10^9 per liter.

16. A method as in claim 1 wherein the subject's reticulocyte count is greater than 120×10^9 per liter.

17. A method as in claim 1 wherein the subject's reticulocyte count is greater than 150×10^9 per liter.

18. A method as in claim 1 wherein said subject has a greater than 40% PNH type III granulocyte clone.

19. A method as in claim 1 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

20. A method of reducing abdominal pain in a subject comprising administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

21. A method as in claim 20 wherein the step of administering comprises administering an anti-C5 antibody.

22. A method as in claim 20 wherein the step of administering comprises administering an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.

23. A method as in claim 20 wherein the step of administering comprises administering a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

24. A method as in claim 20 wherein the step of administering comprises administering a compound selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

25. A method as in claim 20 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 10%.

26. A method as in claim 20 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 25%.

27. A method as in claim 20 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 50%.

28. A method as in claim 20 wherein the subject's platelet count is greater than 40,000 per microliter.

29. A method as in claim 20 wherein the subject's platelet count is greater than 75,000 per microliter.

30. A method as in claim 20 wherein the subject's platelet count is greater than 150,000 per microliter.

31. A method as in claim 20 wherein the subject's reticulocyte count is greater than 80×10^9 per liter.

32. A method as in claim 20 wherein the subject's reticulocyte count is greater than 120×10^9 per liter.

33. A method as in claim 20 wherein the subject's reticulocyte count is greater than 150×10^9 per liter.

34. A method as in claim 20 wherein said subject has a greater than 40% PNH type III granulocyte clone.

35. A method as in claim 20 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

36. A method of treating a hemolytic disease in a subject comprising administering to a subject having a hemolytic disease a compound selected from the group consisting of the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components, wherein there is a reduction in hemoglobinuria in the subject.

37. A method as in claim 36 wherein the compound is selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

38. A method as in claim 36 wherein the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

39. A method as in claim 34 wherein said subject has a greater than 40% PNH type III granulocyte clone.

40. A method as in claim 36 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

41. A method of restoring nitric oxide (NO) homeostasis comprising administering to a subject having a hemolytic disease a compound selected from the group consisting of

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compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

42. A method as in claim 41 wherein the step of administering comprises administering a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

43. A method as in claim 41 wherein the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

44. A method as in claim 41 wherein said subject has a greater than 40% PNH type III granulocyte clone.

45. A method as in claim 41 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

46. A method of reducing hemolysis in a subject having a hemolytic disease comprising administering to the subject a compound selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components, wherein hemolysis is reduced as evidenced by a greater than 50% reduction in lactate dehydrogenase (LDH) levels in the subject's bloodstream.

47. A method as in claim 46 wherein the step of administering comprises administering a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

48. A method as in claim 46 wherein the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

49. A method as in claim 46 wherein said subject has a greater than 40% PNH type III granulocyte clone.

50. A method as in claim 46 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

51. A method of reducing hemoglobinuria in a subject comprising administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

52. A method as in claim 51 wherein the step of administering comprises administering a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

53. A method as in claim 51 wherein the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra 5 venom factor, FUT-175, complestatin, and K76 COOH.

54. A method as in claim 51 wherein said subject has a greater than 40% PNH type III granulocyte clone.

55. A method as in claim 51 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

56. A method of reducing dysphagia in a subject comprising administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

57. A method as in claim 56 wherein the step of administering comprises administering a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

58. A method as in claim 56 wherein the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

59. A method as in claim 56 wherein said subject has a greater than 40% PNH type III granulocyte clone.

60. A method as in claim 56 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

61. A method of reducing erectile dysfunction in a subject comprising administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

62. A method as in claim 61 wherein the step of administering comprises administering a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

63. A method as in claim 61 wherein the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

64. A method as in claim 61 wherein said subject has a greater than 40% PNH type III granulocyte clone.

65. A method as in claim 61 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

66. A method of reducing thrombosis in a subject comprising administering a compound to a subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

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67. A method as in claim 66 wherein the step of administering comprises administering a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

68. A method as in claim 66 wherein the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

69. A method as in claim 66 wherein said subject has a greater than 40% PNH type III granulocyte clone.

70. A method as in claim 66 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

71. A method of increasing proportion of type III red blood cells of a subject's total red blood cell content comprising administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

72. A method as in claim 71 wherein the step of administering comprises administering a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

73. A method as in claim 71 wherein the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

74. A method as in claim 71 wherein said subject has a greater than 40% PNH type III granulocyte clone.

75. A method as in claim 71 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

76. A method of reducing hemolysis in a subject comprising administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

77. A method as in claim 76 wherein the step of administering comprises administering a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

78. A method as in claim 76 wherein the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

79. A method as in claim 76 wherein said subject has a greater than 40% PNH type III granulocyte clone.

80. A method as in claim 76 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

81. A method of treating a nitric oxide (NO) deficiency in a subject afflicted with a hemolytic disease comprising administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

82. A method as in claim 81 wherein the step of administering comprises administering a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

83. A method as in claim 81 wherein the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

84. A method as in claim 81 wherein said subject has a greater than 40% PNH type III granulocyte clone.

85. A method as in claim 81 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

86. A method of rendering a subject afflicted with a hemolytic disease transfusion independent comprising administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

87. A method as in claim 86 wherein the step of administering comprises administering a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

88. A method as in claim 86 wherein the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

89. A method as in claim 86 wherein the subject is transfusion independent for over six months from the first administration of said compound.

90. A method as in claim 86 wherein the subject is transfusion independent for over twelve months from the first administration of said compound.

91. A method as in claim 86 wherein said subject has a greater than 40% PNH type III granulocyte clone.

92. A method as in claim 86 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

93. A method of treating a subject afflicted with a hemolytic disease comprising administering: one or more first compounds selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more

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complement components and compounds which block the activity of one or more complement components; in combination with one or more second compounds that increase hematopoiesis.

94. A method as in claim 93 wherein the step of administering comprises administering a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

95. A method as in claim 93 wherein the first compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

96. A method as in claim 93 wherein the one or more second compounds that increases hematopoiesis is selected from the group consisting of steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin (EPO), antithymocyte globulin (ATG) and antilymphocyte globulin (ALG).

97. A method as in claim 93 wherein said subject has a greater than 40% PNH type III granulocyte clone.

98. A method as in claim 93 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

99. A method of reducing hemolysis in a subject having a hemolytic disease comprising administering to the subject a compound selected from the group consisting of compounds which bind to one or more complement components, compounds which block the geueration of one or more complement components and compounds which block the activity of one or more complement components, wherein hemolysis is reduced as evidenced by a reduction in lactate dehydrogenase (LDH) levels in the subject's bloodstream to within 20% of the upper limit of normal.

100. A method as in claim 99 herein the step of administering comprises administering a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

101. A method as in claim 99 wherein the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

102. A method as in claim 99 wherein said subject has a greater than 40% PNH type III granulocyte clone.

103. A method as in claim 99 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

104. A method of reducing hemolysis in a subject having a hemolytic disease comprising administering to the subject a compound selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components, wherein serum complement hemolytic activity is reduced at least 80% as evidenced by a serum hemolytic assay.

105. A method as in claim 104 wherein the step of administering comprises administering a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

106. A method as in claim 104 wherein the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

107. A method as in claim 104 wherein said subject has a greater than 40% PNH type III granulocyte clone.

108. A method as in claim 104 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

109. A method of decreasing the frequency of transfusions required by a subject afflicted with a hemolytic disease comprising administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

110. The method of claim 109 wherein the frequency of transfusions is decreased by about 50%.

111. The method of claim 109 wherein the frequency of transfusions is decreased by about 70%.

112. The method of claim 109 wherein the frequency of transfusions is decreased by about 90%.

113. A method as in claim 109 wherein the step of administering comprises administering an anti-C5 antibody.

114. A method as in claim 109 wherein the step of administering comprises administering an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.

115. A method as in claim 109 wherein the step of administering comprises administering a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

116. A method as in claim 109 wherein the step of administering comprises administering a compound selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

117. A method as in claim 109 further comprising the step of administering one or more compounds that increase hematopoiesis in combination with said compound.

118. A method as in claim 117 wherein the one or more compounds that increase hematopoiesis is selected from the group consisting of steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin (EPO), antithymocyte globulin (ATG) and antilymphocyte globulin (ALG).

119. A method as in claim 117 wherein EPO is administered in combination with an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.

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120. A method as in claim 109 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 10%.

121. A method as in claim 109 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 25%.

122. A method as in claim 109 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 50%.

123. A method as in claim 109 wherein the subject's platelet count is greater than 40,000 per microliter.

124. A method as in claim 109 wherein the subject's platelet count is greater than 75,000 per microliter.

125. A method as in claim 109 wherein the subject's platelet count is greater than 150,000 per microliter.

126. A method as in claim 109 wherein the subject's reticulocyte count is greater than 80×10^9 per liter.

127. A method as in claim 109 wherein the subject's reticulocyte count is greater than 120×10^9 per liter.

128. A method as in claim 109 wherein the subject's reticulocyte count is greater than 150×10^9 per liter.

129. A method as in claim 109 wherein said subject has a greater than 40% PNH type III granulocyte clone.

130. A method as in claim 109 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

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09. Aug. 2017

Zur Kasse
185

Notice of opposition to a European patent

I. Patent opposed

Patent No.	2 359 834
Application No.	11 001 632.6
Date of mention of the grant in the European Patent Bulletin (Art. 97(3), Art. 99(1) EPC)	
Title of the invention	Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement

II. Proprietor of the patent
first named in the patent specification

	Alexion Pharmaceuticals, Inc.
Opponent's or representative's reference (max. 15 keystrokes)	198 441 u6/ne

III. Opponent

Name	Hoffmann Eitle Patent- und Rechtsanwälte PartmbB
Address	Arabellastr. 30 81925 Munich (DE)
State of residence or of principal place of business	DE
Nationality	
Telephone/Fax	
Multiple opponents (see additional sheet)	☐

IV. Authorisation

1. Representative (name only one representative or name of association of representatives to whom notification is to be made)	Hoffmann Eitle Patent- und Rechtsanwälte PartmbB
Address of place of business	Arabellastraße 30 81925 Munich Germany Association No. 151
Telephone/Fax	089 / 92409-0 | 089 / 918356
Additional representative(s) on additional sheet/see authorisation	☐

Opponent's reference
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2. Name(s) of employee(s) of the opponent authorised to act in these opposition proceedings under Art. 133(3) EPC

Authorisation(s) to 1./2.

not considered necessary [x]

has/have been registered under No. [ ]

is/are enclosed [ ]

V. Opposition is filed against

- the patent as a whole [x]
- 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) [x]
- patentability is excluded on other grounds, i.e. Article [ ] Art.

(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 a separate sheet (annex 1) [X]

VIII. Other requests:

Oral proceedings if the maintenance of the patent amended or unamended is considered.

Opponent's reference
198 441 u6/ne

IX. Evidence presented

Evidence is enclosed [x]

will be filed at a later date [ ]

A. Publications:

1

Particular relevance (page, column, line, fig.):

B: EP 2 359 834 B1 (opposed patent)

2

Particular relevance (page, column, line, fig.):

A: WO 2007/106585 A1 (application as filed)

3

Particular relevance (page, column, line, fig.):

C: US 60/783,070 (priority application)

4

Particular relevance (page, column, line, fig.):

D1: US 2005/0191298 A1

5

Particular relevance (page, column, line, fig.):

D3: Hillmen et al., N Engl. J Med 2004; 350: 552-559

6

Particular relevance (page, column, line, fig.):

D7: US 6,355,245 B1

Continued on additional sheet [x]

B. Other evidence

Continued on additional sheet [ ]

Opponent's reference
198 441 u6/ne

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X. Payment of the opposition fee is made

- as indicated in the enclosed voucher for payment of fees and costs (EPO Form 1010) [x]
- via EPO Online Services [ ]

XI. List of documents

Enclosure No.

0	Form for notice of opposition	[X]	
1	Facts (see VII.)	[X]	
2	Copies of documents presented as evidence (see IX.)		
	a Publications	[x]	
	b Other documents	[ ]	
3	Signed authorisation(s) (see IV.)	[ ]	
4	Voucher for payment of fees and costs (see X.)	[x]	
5	Cheque	[ ]	
6	Additional sheet(s)	[x]	Number of sheets: 1
7	Other	[x]	

Please specify here:

- receipt form

XII. Signature of opponent or representative

Place: Munich

Date: Aug. 09, 2017

Signature: Leo Polz

Name (block capitals): DR. L. POLZ
Hoffmann Eitle PartmbB, Association No. 151

In case of legal persons, signatory's position within company:

Opponent's reference: 198 441 u6/ne

Zusatzblatt / Supplementary Sheet

IX. A. Publications (cont.)

7 D8: Thomas et al., Molecular Immunology, Vol. 33, No. 17/18, 1389-1401, 1996

8 D9: US 2003/0202972 A1

9 D10: Wang et al., J. pharm. Sci., vol. 96, no. 1, p. 1-26, Jan. 2007

10 D11: US 6,171,586 B1

11 D12: Maloney et al., Blood, vol. 90, no.6, 1997: pp 2188-2195

12 ANNEX I: Commercial monoclonal antibodies 2006 (all modes of administration)

13 ANNEX II: Commercial monoclonal antibodies 2006 (i.v. administration)

DR. L. POLZ
Hoffmann Eitle PartmbB, Association No. 151

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HOFFMANN EITLE

EPO - Munich
101
09. Aug. 2017

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Munich, August 09, 2017

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Hoffmann Eitle's professionals include:
European Patent Attorneys
Patentanwälte | German Patent Attorneys
Rechtsanwälte | German Attorneys at Law
British Patent Attorneys (regulated by IPReg)
Italian Patent Attorneys
Spanish Patent Attorneys
Dutch Patent Attorneys
Belgian Patent Attorneys
European Trademark Attorneys
European Design Attorneys

Our Ref.:	**198 441 u6/ne** **Eculizumab Composition / ALEXION**
Opposition to:	**EP 2 359 834 B1 (11001632.6)**
Proprietor:	**Alexion Pharmaceuticals, Inc.** **Cheshire, CT 06410 (US)**
Opponent:	**Hoffmann Eitle** **Patent- und Rechtsanwälte PartmbB** **Arabellastr. 30** **81925 Munich (DE)**

NOTICE OF OPPOSITION

Partnerschaftsregister Amtsgericht München | PR 1372
Eine Liste der Partner i.S.d. PartGG ist einsehbar unter www.hoffmanneitle.com/partner, über die obige Adresse und unser Büro in London:
A list of members of the partnership is open to inspection at www.hoffmanneitle.com/partner, at the above address and our London office:
Hoffmann Eitle | Harmsworth House | 13–15 Bouverie Street | London EC4Y 8DP

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In the name and on behalf of

Hoffmann Eitle
Patent- und Rechtsanwälte PartmbB
Arabellastr. 30
81925 Munich (DE)

(hereinafter also referred to as the "***Opponent***")

we hereby lodge an

OPPOSITION

pursuant to **Art. 99 EPC** to

EP 2 359 834 B1

assigned to

Alexion Pharmaceuticals, Inc.
Cheshire, CT 06410 (US)

(hereinafter also referred to as the "***Proprietor***")

entitled:

"Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement".

A debit order to our account in the amount of **€ 785** is enclosed in accordance with **Art. 99(1) EPC** for the payment of the opposition fee.

Our requests and the statement of the grounds of the opposition are set out below.

REQUESTS

On the grounds of **Art. 100(a) EPC** it is petitioned that:

(1) the opposed patent **EP 2 359 834 B1** be revoked, and
(2) the parties be summoned to attend oral proceedings in accordance with **Art. 116(1) EPC** in the event that the Opposition Division is not in a position to immediately grant the request under (1).

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REASONS

(1) The claimed teaching according to **EP 2 359 834 B1**, hereinafter also referred to as the "***opposed patent***", does not comply with **Art. 56 EPC**, i.e. it does not involve an inventive step.

(2) References to the specification of the opposed patent or to other patent documents will be made – where appropriate – by citing the relevant paragraphs using their corresponding [4-digit] section numbers.

1. CITED PRIOR ART

(3) The present opposition is based on the following documents:

A	**WO 2007/106585 A1** (application as filed)
B	**EP 2 359 834 B1** (opposed patent)
C	**US 60/783,070** (priority application)
D1	**US 2005/0191298 A1**
D2	**Hillmen et al.**, British Journal Haematol., vol.121, Suppl. 1, p.87, **2003**
D3	**Hillmen et al.**, N Engl. J Med **2004**; 350: 552-559
D4	**Harding,** Drugs of the Future, Prous Science, ES, vol.29, no.7, 673-676, **2004**
D5	**US 2005/221382 A1**
D6	**Hill**, Clinical Advances in Hematology & Oncology, Millennium Medical Pub., New York, US, vol.3, no.11, 849-850, **2005**
D7	**US 6,355,245 B1**
D8	**Thomas et al.,** Molecular Immunology, Vol. 33, No. 17/18, 1389-1401, **1996**
D9	**US 2003/0202972 A1**
D10	**Wang et al., J. pharm. Sci., vol. 96, no. 1, p. 1-26, Jan. 2007**
D11	**US 6,171,586 B1**
D12	**Maloney et al.**, Blood, vol. 90, no.6, **1997**: pp 2188-2195

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ANNEX I Commercial monoclonal antibodies 2006 (all modes of administration)
ANNEX II Commercial monoclonal antibodies 2006 (i.v. administration)

(4) Documents **D1** to **D6** correspond to documents D1 to D6 as referenced in the **European Search Report** & **Opinion** dated July 12, 2011. Only **D1** and **D3** are refiled with this notice of opposition, as they will be dealt with in more detail below.

(5) Documents **D7 to D12**, are new documents introduced herewith for the first time into the proceedings.

2. THE OPPOSED PATENT

(6) The original application (**A**) was filed on March 15, 2007 and published as **WO 2007/106585 A1** on September 20, 2007. The opposed patent (**B**) was filed as divisional application **11001632.6** from parent application **07753249.7** and was granted on Nov. 9, 2016

(7) The opposed patent claims the priority of **US 60/783,070** (**C**) filed on March 15, 2006.

2.1 TECHNICAL OBJECTIVE OF THE OPPOSED PATENT

(8) The opposed patent relates the treatment of patients afflicted with ***paroxysmal nocturnal hemoglobinuria***, also referred to as "***PNH***" (cf. [0001]).

(9) "***Eculizumab***" is a humanized monoclonal antibody directed against the terminal complement protein C5. It was shown that eculizumab is able to reduce intravascular hemolysis when the antibody was administered intravenously to PNH patients (cf. [0003]). The results of a follow-up phase III study ("*TRIUMPH*") indicated that any treatment that decreases hemolysis in a patient would result in an improvement in the "*quality of life*" of said patient (cf. [0006]).

(10) The opposed patent seeks to provide a pharmaceutical composition for use in treating a patient afflicted with PNH.

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2.2 TECHNICAL SOLUTION AS CLAIMED

(11) As the technical solution to the above problem, the opposed patent was granted on the basis **claim 1**, which the sole claim of the patent. It reads:

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

2.2.1 Feature Analysis of Claim 1

(12) Accordingly, the pharmaceutical composition of Claim 1 is characterized by following technical features **1** to **7**:

1	*Medical use of the composition is for treating a patient afflicted with **PNH***
2	*the active ingredient of the composition is **eculizumab***
3	*the composition is a solution of a **300 mg eculizumab single-use dosage form***
4	*the **eculizumab concentration** in the composition is **10 mg / ml***
5	*the total **volume** of the composition is **30 ml***
6	*the solution is **sterile***
7	*the solution is **preservation-free***

2.2.2 Volume implicitly follows from of antibody concentration and antibody amount

(13) Notably, claim 1 stipulates that the total volume of the single-use dosage form is **30 ml** (feature 5). This feature, however, directly follows from features 3 and 4: Accordingly, a composition comprising 300 mg eculizumab at a concentration of 10 mg / ml would have a volume of 30 ml, as can be readily figured out by simple mathematical calculation.

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2.2.3 Mode of administration is not limited

(14) The pharmaceutical composition of claim 1 is not restricted to any particular mode of administration. According to paragraph [0058] of the description, administration of the antibodies will generally be performed by an intravascular route, e.g. via intravenous infusion by injection. However, "*other routes of administration may be used if desired*". These routes would include, for example, subcutaneous or intraperitoneal administration.

2.2.4 Method of manufacture is not limited

(15) It is further noted that opposed patent does not provide any specific method how to manufacture the pharmaceutical composition according to claim 1. The only information in this regard can be found in paragraph [0063] merely referring to prior art references, for example ***US 20030202972, US 20040091490, US 20050158316 WO 03/106644, WO 04/066957***, and ***WO 04/091658***.

(16) In this regard, reference is made to **US 2003/0202972**, provided herewith as **D9.** According to [0008] of **D9**, a lyophilized formulation can be reconstituted to generate a stable reconstituted formulation. For specific routes of administration, such as intravenous administration, concentrations of from about "***10 – 40 mg/ml protein in the reconstituted formulation***" are suggested (cf. [0107]).

(17) Hence, the antibody composition of opposed patent can be prepared and stored not only in liquid form, but also in lyophilized form, which is just reconstituted to a liquid solution with the features of claim 1 shortly before administration to the subject, for example, by intravenous infusion.

2.3 No Priority

(18) During the examination procedure, the proprietor relied, in particular, on page 5, lines 8-9, page 6, lines 21-23, and page 22, lines 12-14, of the application as filed (**A**) as basis for claim 1 of the granted patent (cf., for example, submission of January 13, 2016; p.2). These passages, however, are completely missing from the priority

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document **US 60/783,070 (C)**. In fact, there is no disclosure whatsoever on a specific eculizumab formulation in the priority application.

(19) It follows that the opposed patent does not validly claim the priority of **US 60/783,070**. The relevant date for defining the prior art in accordance with **Art. 54(2) EPC** is the filing date of the original application, i.e. **March 15, 2007**.

3. LACK OF INVENTIVE STEP (ARTICLES 100(A) AND 56 EPC)

(20) Claim 1 of the opposed patent does not involve an inventive step.

3.1 SKILLED PERSON IN THE ART AND COMMON GENERAL KNOWLEDGE

(21) The opposed patent relates to the use of an anti-C5 antibody, specifically the already known antibody eculizumab, for the treatment of PNH. Thus the skilled person was, in general, faced with the task of identifying appropriate dosage forms for proceeding forward with this treatment.

(22) The exercise of developing a suitable dosage form is the rudimentary basis to any clinical program. Such a program involves at least a formulation specialist, who is familiar with the manufacture of protein-containing, in particular antibody-containing compositions for administration to a human subject.

(23) This skilled practitioner may seek support, as necessary, from a clinician who is ultimately responsible for the clinical care of the patients (e.g. diagnosis, examination and determination treatment), or from a clinical pharmacologist, who provides dose and frequency recommendations on the basis of pharmacokinetics and pharmacodynamics.

(24) Said person skilled in the art, or team of experts, is possessed of average knowledge and ability. He or she is aware of what was common general knowledge in the art at the relevant date (cf., for example, **T 4/98, T 143/94** and **T 426/88**). Articles in technical journals can be representative of common general knowledge (cf. **T**

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595/90). This applies in particular to articles providing a **review** or **survey** of a topic (cf. **T 309/88**).

(25) The skilled person in the field of pharmaceutical drug development would have been aware of the common general knowledge, as reflected, for example, by **Wang et al. (2007)** (**D10**). This document was published in January 2007 and belongs to the state of art in accordance with **Art. 54(2) EPC**, since the opposed patent can only claim its actual filing date of March 15, 2007 (cf. point 2.3 above).

(26) **D10** reflects on pharmaceutical antibody formulations that have been approved by the health authorities before the filing date of the opposed patent. It is a **review article** and therefore belongs to the common general knowledge in the sense of **T 309/88**. As stated in the Abstract, by 2006 more than **23 monoclonal antibodies** have entered the US market. An overview on the properties of these commercial antibody products, such as administration route or antibody concentration, is given in **Table 1** of **D10**.

(27) It is readily presumed that a **formulation specialist would have consulted D10 for guidance when tasked with the manufacture of a therapeutical antibody formulation**.

(28) Document **D10** will be discussed in more detail below with respect to inventive step.

3.2 Closest Prior Art

(29) **US 2005/0101298** (**D1**) and **Hillmen et al. 2004** (**D3**) relate to the treatment of patients with infusions of eculizumab. As demonstrated in detail below, these documents relate to the same technical field (therapeutic use) as the opposed patent and would require the minimum of structural modifications to arrive at the teaching of claim 1 (cf. **T606/89**). Both documents therefore serve as alternative starting points, i.e. as closest prior art, for the assessment of inventive step according to the EPO's established "*problem-and-solution*" approach.

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3.2.1 Disclosure of US 2005/0101298 (D1)

(30) The disclosure of **D1** relates to a method of treating ***PNH*** in mammals (cf., for example, [0012], [0036]), in particular in human ***patients*** afflicted with this disease (cf. [0067]; Examples).

(31) The Examples report on the implementation of a clinical trial to evaluate the effects of an anti-C5 antibody on PNH and symptoms associated therewith (cf. [0081]). The anti-C5 antibody used was ***eculizumab*** (cf. [0082]; Figures).

(32) "***Eculizumab***" is the tradename for the anti-C5 antibody "***h5G1.1-mAb***" (cf. [0012], penultimate line; [0052]). According to section [0052] of **D1**, the **h5G1.1-mAb**, i.e. eculizumab, and methods for manufacturing it were already known from the prior art:

> *Particularly useful anti-C5-antibodies are* ***h5G1.1-mAb****, h5G1.1-scFv and other functional fragments of h5G1.1.* ***Methods for the preparation of h5G1.1-mAb****, h5G1.1-scFv and other functional fragments of h5G1.1* ***are described in U.S. Pat. No. 6,355,245*** *and "Inhibition of Complement Activity by Humanized Anti-C5 Antibody and Single Chains Fv",* ***Thomas et al.,*** *Molecular Immunology, Vol. 33, No. 17/18, pages 1389-1401,* ***1996, the disclosure of which are incorporated herein in their entirety by this reference****. The* ***antibody h5G1.1-mAb is currently undergoing clinical trials under the trade-name eculizumab****.*
> (cf. lower part of [0052]; emphasis added)

(33) **US 6,355,245** and **Thomas et al. 1996** are enclosed with this opposition brief as **D7** and **D8**, respectively. It will be appreciated, however, that according to the above quotation from [0052], both documents shall be regarded as incorporated in **D1** "*in their entirety*". That means that for applying the problem-and-solution approach the entire disclosure of **D1**, **D7** and **D8** **in combination** represent the "closest prior art"

(34) The mode of administration of eculizumab to a patient suggested by **D1** is, *inter alia*, "*via intravenous infusion*" (cf. [0060]) in a variety of "*unit dosage forms*" (cf. [0058]). The actual dosage regimen used in said clinical trial is described in [0082]:

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*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.*

(35) In summary, **D1** discloses the treatment of PNH patients with a pharmaceutical composition comprising eculizumab, said pharmaceutical composition being suitable for intravenous infusion.

3.2.2 Disclosure of Hillmen et al. 2004 (D3)

(36) **D3** is a scientific publication corresponding to the clinical study mentioned in the Examples of **D1**. It describes the details of the procedure and the results of the study in more detail. In particular, the clinical efficacy of eculizumab was tested in patients with PNH (cf. "*Background*"). Eleven transfusion-dependent patients with PNH received infusions of eculizumab (600mg) 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 (cf. "*Methods*").

Accordingly, **D3** basically comprises the same disclosure as **D1** and could likewise serve as the closest prior art. For the sake of convenience, the following assessment will be based on **D1** as the closest prior art.

3.3 OBJECTIVE TECHNICAL PROBLEM

(37) As outlined above, **D1** (or **D3**) disclose a *pharmaceutical composition for use in treating a patient afflicted with PNH* in the clinical study of the Examples. The active ingredient is eculizumab.

(38) Hence, features 1 and 2 of present claim 1 are disclosed in **D1**. Since the eculizumab comprising composition of **D1** is to be administered via intravenous infusion, it must be constituted as an aqueous solution.

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3.3.1 Feature comparison of Claim 1 with D1

(39) **D1** does not particularly specify how the composition is formulated, especially in terms of the absolute amount and concentration of eculizumab in the dosage form.

(40) As compared to **D1**, features 3 to 7 of granted claim 1 provide concrete characteristics of the pharmaceutical composition of the opposed patent. These are:

- the **total amount** of eculizumab in the single-use dosage form is **300 mg** (feature 3),
- the eculizumab **concentration** is **10 mg / ml** (feature 4),
- the **volume** is **30 ml** (feature 5), and
- the solution is **sterile** (feature 6), and
- the solution is **preservative-free** (feature 7).

3.3.2 No technical effect

(41) Features 1 to 7 in combination, in particular with regard to features 3 to 7, are described in sections [0017] and [0057] of the patent specification.

(42) No technical effects or advantages whatsoever contributed by said features 3 to 7, in terms of, for example, stability of the liquid formulation or shelf-life, are disclosed or merely suggested in the patent specification. No comparative experiments with other administration forms had been performed.

(43) Notably, the section "EXEMPLIFICATION" in the opposed patent, which describes the "TRUMPH" clinical study, only specifies the total amount of eculizumab (600 mg or 900 mg) administered to the patients via intravenous infusion (cf. [0074]), but is silent on the other features 3 to 7 of the pharmaceutical composition, such as eculizumab concentration in the solution etc.. Thus, it remains unclear whether the eculizumab formulation as claimed was actually used in the Examples of the opposed patent.

(44) In summary, when reading the specification as a whole, and in particular paragraphs [0063] to [0069] it becomes obvious that the **inventors considered the specific features of the eculizumab formulation rather conventional**. There is no suggestion

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whatsoever that there might be a surprising technical effect related to the claimed eculizumab composition, which is neither self-evident nor predictable.

3.3.3 Technical Problem as basis for the problem-and-solution approach

(45) The objective technical problem underlying the opposed patent is therefore to provide a pharmaceutical composition comprising eculizumab for use in the clinical studies described in any of **D1** or **D3**.

(46) Said composition shall be characterized in terms of the absolute amount and concentration of eculizumab, and be suitable for safe administration to human patients.

3.4 CLAIM 1 IS OBVIOUS OVER A COMBINATION OF D1 / D3 IN COMBINATION WITH US 6,171,586 (D11)

(47) In order to solve the objective technical problem of the opposed patent, the skilled person would have readily turned to **D11**.

3.4.1 Teaching of US 6,171,586 (D11)

(48) **D11** generally discloses a *stable aqueous **pharmaceutical formulation** comprising a therapeutically effective amount of an **antibody*** (cf. abstract; claim 1; col.2, l.25-33). In this regard, **D11** points to typical problems in connection with biological proteins like antibodies, such as degradation, chemical and physical instability; the most common protein degradation pathways being protein aggregation, deamination and oxidation (cf. col.1, l.15-38).

(49) The specification of **D11** provides abundant technical teaching how to overcome these problems, for example, by carefully controlling buffer composition, pH, and additives, such as surfactant and polyol. Accordingly, an antibody composition of **D11** is stable at a temperature of about 2 – 8°C for at least one year, and/or is stable at a temperature of about 30°C for at least one month, and/or is stable following freezing and thawing of the formulation (cf. col.2, l.25-34).

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(50) The general principles of this document are not limited to a specific antibody, but are rather applicable to antibodies in general. This becomes clear already from the formulation of the technical object underlying **D11**:

> *There is a need in the art for a **stable aqueous pharmaceutical formulation comprising an antibody,** such as an anti-CD18 or anti-CD20 antibody, which is **suitable for therapeutic use.***
> (cf. col.2, l.19-22; emphasis added)

(51) The antibodies for incorporation into the stable pharmaceutical composition of **D11** can be directed to a vast number of highly different protein antigens. A non-limiting list of such antigens is provided in column 10, lines 1 to 67 of the specification. The wording "*such as*" in the above quotation makes it clear that the specific anti-CD18 or anti-CD20 antibodies just represent exemplary embodiments of suitable antibodies. The skilled person would understand that the examples of **D11** dealing with anti CD18 and CD20 antibodies just serve to demonstrate the applicability of the invention of **D11**, but would not be construed as limiting the scope of the invention in terms of the antibody specificity (cf. col.24, l.22-26).

(52) Further, the antibodies of **D11** are not limited to specific classes or types of monoclonal antibodies, including antibody fragments, chimeric or "*humanized*" forms (cf. col.8, in particular l.54). Even if not specifically mentioned in the specification, the person skilled in the art would readily understand that also eculizumab could equally well be processed into a stable and safe pharmaceutical composition applying the methods of **D11**.

(53) **D11** also relates to containers holding said pharmaceutical formulation (cf. col.2, l.34-39). Suitable containers include, for example, bottles and vials formed from glass or plastic. Exemplary containers may 3 to 100 ml glass vials (cf. col.24, l.7-21).

(54) It therefore follows that the skilled person was clearly **motivated** to turn to **D11** for guidance when faced with the task of providing a pharmaceutical antibody formulation for use in the clinical study of **D1**. In doing so, the skilled person would have arrived at the pharmaceutical antibody composition according to claim 1 without any inventive effort, as outlined below:

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3.4.2 Total amount of 300 mg eculizumab in a single use-dosage form (feature 3)

(55) In the clinical study of **D1**, patients received two distinct doses of the active ingredient: First, **600 mg** doses of eculizumab were intravenously administered every week for four weeks. Then doses of **900 mg** were administered every other week.

(56) It is immediately obvious to the skilled person that the composition should be formulated as a **single-use dosage form comprising 300 mg eculizumab** (feature 3). This would allow conveniently using one and the same dosage form for both regimens, i.e. **two** single-use dosage forms for the 600 mg administration, and **three** single-use dosage forms for the 900 mg administration.

3.4.3 Eculizumab concentration of 10 mg / ml (feature 4)

(57) For the clinical study of **D1**...

> *.... each of 11 patients received a weekly **600 mg intravenous infusion** of anti-C5 antibody for approximately **thirty minutes**.*
> (cf. **D1**, [0082]; emphasis added)

(58) The skilled person was therefore set to the task to select a concentration of the antibody and a volume such that two single-use dosage forms of the pharmaceutical formulation each comprising 300 mg antibody can be intravenously administered within a period of thirty minutes.

(59) In order to implement the specific concentration of antibody / volume, the skilled person would have found further guidance in documents relating to antibody formulations for pharmaceutical applications, for example, **D11**.

(60) Accordingly, the antibody concentration in the formulations of **D11** is typically from about 0.1 to about 50 mg / ml (cf. claim 13). Preferred antibody concentrations are mentioned in column 22, lines 13 to 17 of the specification:

> *From about 0.1 mg / ml to about 50 mg / ml, preferably, from about 0.5 mg / ml to about 25 mg / ml, and **most preferably** from 2 mg / ml to*

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> *about **10 mg / ml** is an exemplary antibody concentration in the formulation (cf. col. 22, l.13-17).*
> (emphasis added)

(61) The above passage is quoted from the general description of **D11** and does not relate to any antibody in particular. It would therefore, *prima facie*, be applicable to any antibody. It should be particularly emphasized that the antibody concentration of "***10 mg / ml***" is **expressly** mentioned in **D11** as belonging to the **most preferred** concentration range.

(62) It was obvious to the skilled person with a reasonable expectation of success that the antibody concentrations mentioned in **D11** would be also applicable for a pharmaceutical eculizumab formulation. Feature 4 relating to the antibody concentration of 10 mg / ml is therefore an entirely obvious choice.

(63) Regarding the general applicability of **D11** with respect to different antibodies, and regarding the skilled person's expectation that the most preferred antibody concentrations suggested in **D11**, in particular 10 mg / ml, would have successfully worked also for eculizumab, see also point 3.5 below.

3.4.4 Total volume 30 ml (feature 5)

(64) As explained above, the volume of the composition implicitly, but nevertheless directly and unambiguously, follows from the absolute amount (300 mg; feature 3) and the concentration of the active ingredient in the solution (10 mg / ml; feature 4). Since both features 3 and 4 are obvious as demonstrated above, also feature 5 relating to the volume of 30 ml is obvious (300 mg / (10 mg / ml) = 30 ml).

3.4.5 Solution is sterile (feature 6)

(65) It goes without saying that formulations to be used for *in vivo* administration to a human subject must be sterile. This is common general knowledge and is, of course, appreciated by **D11**, too:

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> *The formulations to be used for in vivo administration* ***must*** *be* ***sterile****. This is readily accomplished by filtration through* ***sterile*** *filtration membranes, prior to, of following preparation of the formulation.*
> (cf. col. 23, l.27-30; emphasis added)

(66) Feature 6 does therefore not make any inventive contribution.

3.4.6 Solution is preservative-free (feature 7)

(67) According to the original application of the opposed patent, the pharmaceutical antibody compositions **may** or **may not** include preservatives (cf. **A**, for example, p.6, l.20, and p.24, l.23). Notably, even in the specification of the patent as granted it is still mentioned that preservatives can be included in the composition, leaving some doubts on the scope of granted claim 1:

> *Wetting agents, emulsifiers and lubricants, [.....] and perfuming agents,* ***preservatives*** *and anti-oxidants* ***can also be present*** *in the pharmaceutical compositions of the application.*
> (cf. [0064]; emphasis added)

(68) A similar disclosure can be found in paragraph [0058] of the opposed patent, stating that the "*formulations may contain pharmaceutically acceptable auxiliary substances as required, such as [....],* ***preservatives****, [....], and the like*".

(69) It is important to emphasize that no specific technical effect has been demonstrated in regard of the absence of preservatives. The limitation to "*preservative-free*" was therefore an obvious choice without any inventive contribution.

(70) In any event, **D11** expressly discloses the feature "*preservative-free*" as an alternative to the presence of preservatives, thereby clearly suggesting that preservatives are not necessary to obtain stable pharmaceutical antibody formulations:

> *In one embodiment, the formulation contains the above-identified agents (i.e. antibody, buffer, polyol and surfactant) and is* ***essentially free of one or more preservatives****, such as benzyl alcohol, phenol, m-cresol,*

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chlorobutanol and benzethonium C1.
(cf. col. 22, l.62-64; emphasis added).

(71) In conclusion, the teaching of claim 1 does not involve an inventive step.

3.5 CLAIMED ANTIBODY CONCENTRATION IS COMMON GENERAL KNOWLEDGE

(72) The common general knowledge, as reflected, for example, by **D10**, confirms the universal applicability of **D11** for the manufacture of various stable antibody formulations, in particular with respect to the concentration of the antibody of **10 mg / ml**.

3.5.1 Antibody concentrations of D11 are typical for commercial antibody formulations

(73) As mentioned in point 3.4.3 above, the broadest range for the antibody concentration suggested in **D11** is from **0.1 mg / ml to 50 mg / ml**, a preferred range being from **0.5 mg/ml to 25 mg/ml**, and the most preferred range being from **2 mg/ml to 10 mg/ml** (cf. col.22; l.13-17).

(74) Table 1 of **D10** lists **23 commercial therapeutical products** comprising authorized antibodies. The antibody concentration in the formulation for each product is given in the column headed "***Mab Conc***". One can reasonably presume that a skilled person - commissioned to prepare a therapeutical formulation for a new antibody - would have oriented himself on existing commercial antibody formulations, in particular with respect of such a mandatory parameter as the antibody concentration. All the more so because the number of approved antibody at the priority date was only 23, i.e. fairly manageable.

(75) In the table of **ANNEX I** submitted with this opposition, the antibodies of Table 1 of **D10** were grouped according the three concentration ranges of **D11**. That is: if the concentration of a specific antibody formulation fell within the range the broadest range of 0.1 to 50 mg / ml, the box in the corresponding column was checked (5th column of ANNEX I). If the concentration fell into the preferred range of 0.5 mg / ml – 25 mg / ml, the box in this column (6th col.) was checked, too, and so forth. In case

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the antibody concentration was exactly 10 mg / ml, the box in the last column (10 mg / ml) was checked.

(76) Since the antibody formulation of the present invention is not limited to any mode of administration, also routes other than intravenous administration were considered. Moreover, since the formulation according to claim 1 can also be reconstructed from a lyophilized powder formulation, such commercial products were likewise included.

(77) As one can take from column 5 of **ANNEX I**, 20 out of 23 therapeutic antibody formulations **(87%)** have a concentration within the range of 0.1 to 50 mg / ml. Still 18 of 23 antibody concentrations **(78%)** fall within the preferred range of 0.5 to 25 mg / ml, and 9 out of 23 antibody concentrations **(39%)** are within the most preferred range of **D11**, i.e. 2 to 10 mg / ml. Most remarkably, the number of commercial antibody formulations having a concentration of exactly **10 mg / ml** was still as high as **22%** (5 out of 23)!

(78) The individual antibody concentrations of the respective antibodies vary within the above ranges, but it is to be emphasized that 1**0 mg / ml is the most commonly used concentration** (5 formulations, versus 2 formulations with 0.5 mg / ml or 2 mg / ml, respectively).

(79) The clear and straightforward preference for an antibody concentration of 10 mg / ml becomes even more manifest if only antibody formulations for intravenous administration (18 antibody products) are considered. According to **ANNEX II**, 18 out of 18 therapeutic antibody formulations **(100%)** have a concentration within the range of 0.1 to 50 mg / ml. 17 of 18 antibody concentrations **(94%)** fall within the preferred range of 0.5 to 25 mg / ml, and 8 out of 18 antibody concentrations **(44%)** are within the most preferred range of 2 to 10 mg / ml. The number of antibody formulations having a concentration of exactly **10 mg/ml** is still **22%** (4 out of 18).

3.5.2 Preferred antibodies of D11 are commercially used at a concentration of 10 mg / ml

(80) As an antigen of special interest **D11** mentions the **CD20 surface antigen** which can be targeted for treating B cell lymphomas (cf. col.2, l.59-61). Several antibodies

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against CD20 were known at the filing date of **D11**, including the chimeric antibody termed "***C2B8***" (cf. col.2, lines 14-18 & reference to US 5,736,137 by Idec Pharmaceutical Corporation). Idec's C2B8 antibody is also known by the INN (international nonproprietary name) "***rituximab***", see for example **Maloney et al. 1997** (**D12**; ct. title).

(81) The antibody product "**Rituxan**" comprising rituximab is listed among the 23 approved pharmaceutical antibody formulation of **D10**. According to Table 1, no 16, Rituxan comprises "**10 mg / ml**" of antibody in a solution of intravenous administration.

(82) Feature 4 would therefore belong to the common general knowledge.

3.5.3 Concentration of 10 mg / ml was obvious to try

(83) Thus, the skilled person would conclude from these data that the antibody concentrations suggested in **D11**, in particular 10 mg / ml would be generally applicable for many different antibodies. In fact, every 5th approved antibody that was commercialized before the priority date of the opposed patent, was successfully used at exactly the same concentration as claimed in the opposed patent, i.e. 10 mg/ml.

(84) It was therefore obvious for the skilled person to try out this concentration also for eculizumab with a reasonable expectation of success. The selection of 10 mg/ml according to feature 4 does not involve an inventive step.

4. CONCLUSION

(85) It clearly follows from the above that the arbitrary provision of a formulation form is not patentable merely because it potentially "enriches" the already existing repertoire of formulation forms. All of the features in combination as claimed in the opposed patent were obvious over **D1** (or **D3**) in combination with **D11,** or common general knowledge as reflected by **D10**.

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(86) Consequently, the opposed patent does not meet the requirements of the EPC. It has therefore to be revoked *in toto*.

Leo Polz

DR. LEO POLZ

European Patent Attorney
Hoffmann Eitle Patent-und Rechtsanwälte PartmbB
Association number 151

Enclosures:

- Debit order in the amount of **€ 785.00** for the payment of the opposition fee
- Documents under **SECTION 1** above (except for D2, and D4 to D6).

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Page 5987

Document made available under the Patent Cooperation Treaty (PCT)

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Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

Exhibit no. C
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May 21, 2007

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

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

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Page 5989

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET – Page 1 of 2

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

Express Mail Label No. ED 481024650 US

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

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

TITLE OF THE INVENTION (500 characters max):

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

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

☐ The address corresponding to Customer Number:

OR

☑ Firm or Individual Name Stephen A. Saxe

Address Alexion Pharmaceuticals, Inc.
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Cheshire	Connecticut	06410
Country U.S.A.	Telephone 203-271-8289	Email saxes@alxn.com

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

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

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

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

☑ Applicant claims small entity status. See 37 CFR 1.27.
☐ A check or money order is enclosed to cover the filing fee and application size fee (if applicable).
☐ Payment by credit card. Form PTO-2038 is attached
☑ The Director is hereby authorized to charge the filing fee and application size fee (if applicable) or credit any overpayment to Deposit Account Number: 01-0483. A duplicative copy of this form is enclosed for fee processing.

100.00
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031506 17224 U.S.PTO
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Page 2 of 2

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

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SIGNATURE Stephen A. Saxe Date March 15, 2006

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

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

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Page 5991

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FEE TRANSMITTAL
For FY 2006

☑ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 100.00

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

METHOD OF PAYMENT (check all that apply)

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

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

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

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1. BASIC FILING, SEARCH, AND EXAMINATION FEES

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

2. EXCESS CLAIM FEES

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

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

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

Indep. Claims	Extra Claims	Fee ($)	Fee Paid ($)
_____ - 3 or HP =	_____ x	_____ =	0

HP = highest number of independent claims paid for, if greater than 3.

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

3. APPLICATION SIZE FEE

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

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

4. OTHER FEE(S)

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

SUBMITTED BY

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

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TRANSMITTAL FORM

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Total Number of Pages in This Submission: 41

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

ENCLOSURES *(Check all that apply)*

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

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

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

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

CERTIFICATE OF TRANSMISSION/MAILING

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Signature	Stephen a. Saxe		
Typed or printed name	Stephen A. Saxe	Date	March 15, 2006

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Docket No.: 114 CIP2 PRO

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

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

CERTIFICATE OF EXPRESS MAILING

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[x] Provisional Application for Patent Cover Sheet (2 pages)
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[x] Specification (32 pages)
[x] Drawings (4 pages)
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Stephen A. Saxe

Stephen A. Saxe
Alexion Pharmaceuticals, Inc.
352 Knotter Drive
Cheshire, CT 06410
Tel. 203-271-8289

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

BACKGROUND

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

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

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

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SUMMARY

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

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

BRIEF DESCRIPTION OF THE FIGURES

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

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

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

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

DETAILED DESCRIPTION

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

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

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

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

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

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I. METHODS

Patient Selection

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

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

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

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

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

Study Design

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

Measures of Clinical Efficacy

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

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

Safety Assessments

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

Statistical Analysis

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

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

II. RESULTS

Characteristics of Patients

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

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

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

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

Pharmacokinetics/Pharmacodynamics

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

Hemolytic Efficacy Variables

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

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

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

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

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

III. CLINICAL EFFICACY

Co-primary endpoints

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

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

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Table 1

Stabilization of Hemoglobin Levels and Reduction

in Transfusion Requirements during Eculizumab Treatment

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

*Twelve month historical transfusion data normalized to 6 months

†Co-primary endpoints

‡Base on 2-sided Fisher's exact test

§Based on Wilcoxon's rank sum test

NA, not applicable

Improvements in quality of life measures

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

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

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

Table 2

Effect of Eculizumab Treatment on

Quality of Life Assessed by the EORTC QLQ-C30 Instrument

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

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

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

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

Relationship between FACIT-Fatigue quality of life and intravascular hemolysis

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

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Table 3

Relationship between FACIT-Fatigue and Intravascular Hemolysis

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

Safety

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

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

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

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

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Table 4

Adverse Event Reporting

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

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

*By preferred terms

†Occurring in 10% or more of patients

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

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

IV. DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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WHAT IS CLAIMED IS:

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

53. The method of claim 52 wherein said antibody or active antibody fragment is selected from the group consisting of a) polyclonal antibodies, b) monoclonal antibodies, c) single chain antibodies, d) chimeric antibodies, e) humanized antibodies, f) Fabs, g) F(ab')s, h) $F(ab')_2$s, i) Fvs, j) diabodies, and k) human antibodies.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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ABSTRACT

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

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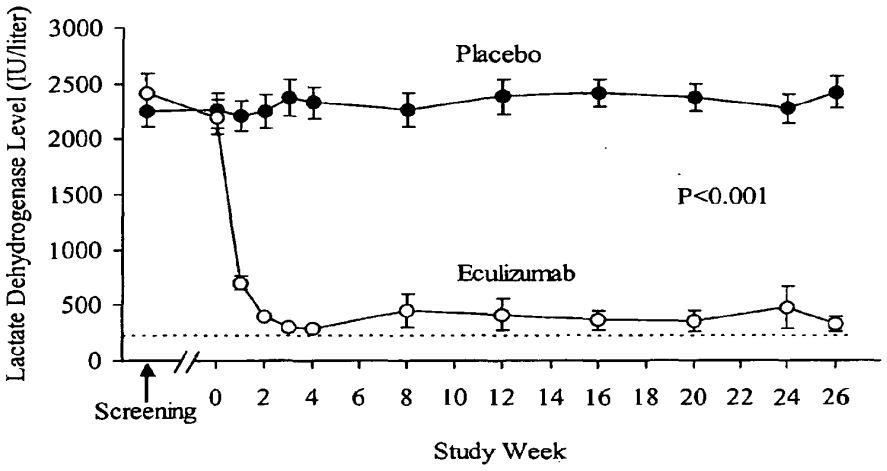


FIGURE 1A

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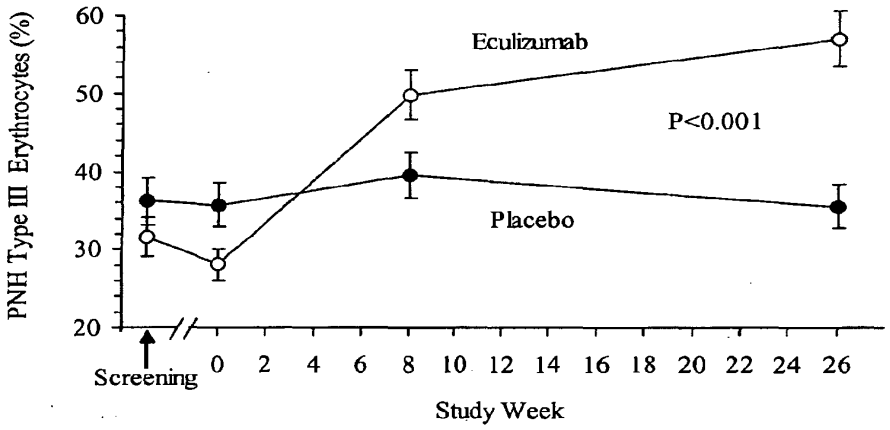


FIGURE 1B

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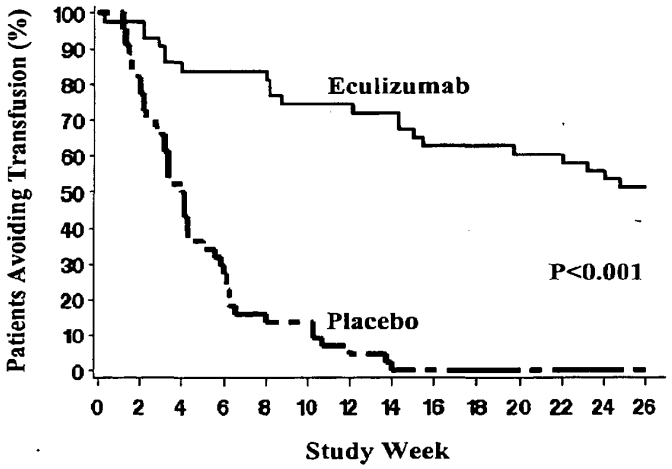


FIGURE 2

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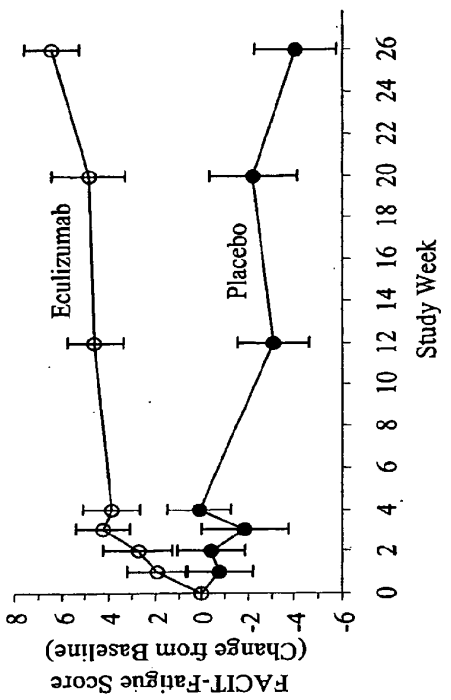


FIGURE 3

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RAPID COMMUNICATION

IDEC-C2B8 (Rituximab) Anti-CD20 Monoclonal Antibody Therapy in Patients With Relapsed Low-Grade Non-Hodgkin's Lymphoma

By David G. Maloney, Antonio J. Grillo-López, Christine A. White, David Bodkin, Russell J. Schilder, James A. Neidhart, Nalini Janakiraman, Kenneth A. Foon, Tina-Marie Liles, Brian K. Dallaire, Ken Wey, Ivor Royston, Thomas Davis, and Ronald Levy

IDEC-C2B8 is a chimeric monoclonal antibody (MoAb) directed against the B-cell–specific antigen CD20 expressed on non-Hodgkin's lymphomas (NHL). The MoAb mediates complement and antibody-dependent cell-mediated cytotoxicity and has direct antiproliferative effects against malignant B-cell lines in vitro. Phase I trials of single doses up to 500 mg/m² and 4 weekly doses of 375 mg/m² showed clinical responses with no dose-limiting toxicity. We conducted a phase II, multicenter study evaluating four weekly infusions of 375 mg/m² IDEC-C2B8 in patients with relapsed low-grade or follicular NHL (Working Formulation groups A-D). Patients were monitored for adverse events, antibody pharmacokinetics, and clinical response. Thirty-seven patients with a median age of 58 years (range, 29 to 81 years) were treated. All patients had relapsed after chemotherapy (median of 2 prior regimens) and 54% had failed aggressive chemotherapy. Infusional side effects (grade 1-2) consisting of mild fever, chills, respiratory symptoms, and occasionally hypotension were observed mostly with the initial antibody infusion and were rare with subsequent doses. Peripheral blood B-cell depletion occurred rapidly, with recovery beginning 6 months posttreatment. There were no significant changes in mean IgG levels and infections were not increased over what would be expected in this population. Clinical remissions were observed in 17 patients (3 complete remissions and 14 partial remissions), yielding an intent to treat response rate of 46%. The onset of these tumor responses was as soon as 1 month posttreatment and reached a maximum by 4 months posttreatment. In the 17 responders, the median time to progression was 10.2 months (5 patients exceeding 20 months). Likelihood of tumor response was associated with a follicular histology, with the ability to sustain a high serum level of antibody after the first infusion, and with a longer duration of remission to prior chemotherapy. One patient developed a detectable but not quantifiable immune response to the antibody that had no clinical significance. IDEC-C2B8 in a dose of 375 mg/m² weekly for 4 weeks has antitumor activity in patients with relapsed low-grade or follicular NHL. Results with this brief, outpatient treatment compare favorably with results with standard chemotherapy, and IDEC-C2B8 has a better safety profile. Further studies evaluating IDEC-C2B8 in other types of lymphoma either alone or combined with chemotherapy are warranted.
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THE TREATMENT OF cancer with agents specific for the tumor cell with sparing of the host has long been a goal of oncology. Unfortunately, treatment approaches using chemotherapy and radiotherapy have increased host toxicity to obtain antitumor activity. The development of monoclonal antibodies (MoAbs) provided hope that tumor-targeted therapy would one day play a role in the treatment of cancer. Indeed, promising results have been presented in several areas; however, these treatments have often proved technically difficult, produced disappointing efficacy, and were often not broadly applicable to patients with a given malignancy. The treatment of patients with lymphoma is an exception. Patients with advanced stage or relapsed low-grade non-Hodgkin's lymphoma (NHL) are not curable using conventional approaches and are usually treated with combination chemotherapy regimens of increasing intensity as needed to reduce disease and palliate symptoms.[1] Aggressive approaches using high-dose therapy and autologous marrow support may provide maximal tumor reduction; however, there is currently no plateau on the failure-free survival curve.[2] New treatment modalities and approaches that provide tumor reduction with less toxicity are needed.

MoAbs against a variety of tumor surface antigens have shown activity in the treatment of patients with low-grade NHL. Unmodified murine MoAbs have included custom, patient-specific anti-idiotype MoAbs,[3-6] anti-CD20 MoAbs,[7] and antibodies against CD19 and CD22 armed with immunotoxins.[8-10] Anti-CD20 MoAbs radiolabeled with ^{131}I or ^{90}Y in low dose[11-13] and in high dose with stem cell support[14,15] have also demonstrated significant antitumor activity in the treatment of patients with relapsed disease, but have encountered dose-limiting toxicities and are limited by bone marrow involvement.

IDEC-C2B8 is a chimeric anti-CD20 MoAb containing human IgG1 and κ constant regions with murine variable regions.[16] It binds the CD20 antigen with high affinity (5×10^{-9} mol/L). Because of the human constant Ig regions, the chimeric antibody efficiently kills CD20$^+$ cells in vitro by augmented complement-mediated lysis and participates in antibody-dependent cell-mediated cytotoxicity (ADCC) using human complement and immune effector cells.[16] In some

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NHL cell lines, the binding of the antibody inhibits proliferation and directly induces apoptosis.[17] In patients with lymphoma, the administration of single doses up to 500 mg/m^2 was not associated with any dose limiting toxicity. Rapid binding to and depletion of CD20$^+$ normal B cells and tumor cells in the peripheral blood and bone marrow was observed, and tumor cells in lymph node biopsies obtained 2 weeks after antibody therapy showed that the chimeric MoAb bound to CD20 antigen sites.[18] The treatment was well tolerated, causing only minimal infusion-related symptoms. Tumor responses (2 partial remissions [PR] lasting 8.1 and 8.6 months and 5 minor responses lasting 0.9 to 6 months) were observed in 7 of 9 patients treated with single doses of at least 100 mg/m^2. Repeated weekly infusion for 4 weeks of doses of 125, 250, or 375 mg/m^2 in 20 patients with relapsed lymphoma has also recently been reported.[19] The treatment was well tolerated and 6 of 18 evaluable patients had a PR with no dose-limiting toxicity identified. The 375 mg/m^2 for 4 weeks dose was selected for phase II evaluation. We report here on the clinical results obtained in the treatment of 37 patients with relapsed low-grade or follicular lymphoma.

MATERIALS AND METHODS

Study design. This was a phase II, open-label, single-arm, multicenter trial evaluating four intravenous infusions of the chimeric monoclonal anti-CD20 antibody IDEC-C2B8 at a dose of 375 mg/m^2 in patients with relapsed low-grade NHL. Study objectives included the evaluation of safety, dose-limiting toxicities, pharmacokinetics, immune response, and clinical activity. Patients received a single infusion weekly, and completed the 4 antibody infusions in 22 days.

Antibody. IDEC-C2B8 is a chimeric MoAb containing human IgG1 (κ) heavy and light chain constant regions and murine variable regions from the murine antihuman CD20 MoAb IDEC-2B8 (murine IgG1, κ). The chimeric antibody was produced in vitro from a transfected Chinese hamster ovary (CHO) cell line.[16] The antibody is specific for the human CD20 antigen, a 35-kD phosphoprotein present on normal and malignant B cells, but not on other tissues.[20] The antibody was produced and supplied by IDEC Pharmaceuticals Corp (San Diego, CA).

Patient population and sample size. Adults with histologically confirmed, relapsed B-cell lymphoma expressing the CD20 antigen were eligible, as were nonresponders to first chemotherapy. All patients met the following criteria: expected survival of $\geq$3 months with no serious nonmalignant disease; prestudy performance status of 0, 1, or 2 on the World Health Organization (WHO) scale; seronegative for human immunodeficiency virus (HIV) and hepatitis B surface antigen; serum IgG level of $\geq$600 mg/dL; hemoglobin level of $\geq$8.0 g/dL; white blood cell count of $\geq 3.0 \times 10^3/\mu L$; absolute granulocyte count of $\geq 1.5 \times 10^3/\mu L$; platelet count of $\geq 75 \times 10^3/\mu L$; bilirubin level of less than 1.5 mg/dL; alkaline phosphatase level of less than 2$\times$ normal; aspartata amino transferase (AST) level of less than 2$\times$ normal; and serum creatinine level of less than 2.0 mg/dL. Patients tested negative for human antimurine antibody (HAMA).

The 375 mg/m^2 dose was identified from a phase I dose-escalation trial evaluating 125, 250, and 375 mg/m^2 infusions for 4 weeks.[19] To determine clinical efficacy, a total of 37 patients were treated at the 375 mg/m^2 dose and form the basis of this report.

Study sites. Patients were treated at seven sites in the United States. All patients signed an informed consent approved by the local Institutional Review Boards at Stanford University Medical Center (Palo Alto, CA), San Diego Regional Cancer Center (San Diego, CA), Scripps Memorial Hospitals (La Jolla and Encinitas, CA), Fox Chase Cancer Center (Philadelphia, PA), University of New Mexico Cancer Center (Albuquerque, NM), Henry Ford Hospital (Detroit, MI), or Markey Cancer Center (Lexington, KY). Patients were enrolled and treated between August 12, 1993 and September 13, 1994.

Pharmacokinetic sampling and HAMA/HACA evaluation. Serum samples were obtained pretreatment and at scheduled follow-up evaluations to determine anti-CD20 MoAb levels and to analyze for evidence of human antichimeric antibody (HACA) and HAMA immune response. Extended pharmacokinetic sampling was performed during the treatment course in selected patients whose schedules permitted. This included samples during the weeks 1 and 4 of treatment, including before infusion, postinfusion, and 24, 48, 72, and 96 hours postinfusion, as well as preinfusion and postinfusion samples from weeks 2 and 3. Serum pharmacokinetics were determined using frozen serum samples on microtiter plates coated with polyclonal goat anti–IDEC-2B8 antibody. Serum samples were serially diluted and washed, and bound IDEC-C2B8 was detected using labeled goat antihuman IgG and compared with a standard curve obtained using IDEC-C2B8 diluted into normal human serum. The assay has a lower limit of quantification of 0.5 μg/mL of the chimeric antibody.

HAMA was detected using microtiter plates coated with murine IgG. Serum samples were serially diluted on the plate and washed. Any bound human Ig was then detected using labeled goat antihuman Ig. HACA was detected by incubating serum samples on plates coated with IDEC-C2B8. Human antibody bound to the microtiter wells was then detected using biotin–IDEC-C2B8 followed by avidin-horseradish peroxidase and the substrate 2, 2-azinobis (3-ethylbenzthiazoline sulfonic acid) (ABTS). The minimum quantifiable level for these assays was 100 ng/mL (detectable to 5 ng/mL).

Patient monitoring and efficacy evaluation. Patients were monitored for safety and for clinical antitumor effect with regular medical histories, physical examinations, and laboratory studies including complete blood count and differential, chemistry panel, quantitative serum Igs, serum complement, serum anti-CD20 levels (HACA and HAMA), and urinalysis performed at baseline; at weeks 1, 2, 3, and 4; and at specified intervals to a maximum of 4 years. Toxicity was evaluated using the National Cancer Institute's Adult Toxicity Criteria. Peripheral blood was analyzed by flow cytometry to determine lymphocyte subsets at baseline, week 1, months 1, and 3 and at 3-month intervals for 1 year. Chest x-ray was performed at baseline and at 1 and 3 months after the completion of IDEC-C2B8 therapy. Computed tomography scans of the neck, chest, abdomen, and pelvis, as well as physical examination and measurement of palpable tumor masses or lymph nodes was conducted at baseline, at 1 and 3 months after treatment, and at 3-month intervals for 1 year and then yearly for 4 years. Tumor lesions were serially measured by physical exam and radiologic procedures. PR were defined as a decrease of greater than 50% in the total sum of the products of the bidimensional measurements. Complete remissions (CR) were defined as the disappearance of all disease. Stable disease was defined by no significant change in tumor measurements without progression over the period of observation. Remissions (PR and CR) were confirmed on a second complete evaluation at least 28 days later. Disease progression was determined by a 25% increase in measurable disease or the appearance of any new lesion. Time to progression was measured from date of first antibody infusion to first date when progressive disease was documented.

RESULTS

Patient characteristics. Thirty-seven patients were assigned to receive treatment at the phase II dose level of 375

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Table 1. Patient Characteristics (N = 37)

	No.	% or Range
Age	58	29-81
Sex		
Female	16	43
Male	21	57
Histologic grade*		
Low	34	92
A	4	11
B	21	57
C	9	24
Intermediate	3	8
D	2	5
G	1	3
Stage at diagnosis		
I	5	14
II	2	5
III	10	27
IV	18	49
Unknown	2	5
Prior Therapy		
Chemotherapy	37	100
Median no. of regimens	2	1-5
Time from last Rx median (mo)	10.2	0.9-70.4
Radiotherapy	13	35
ABMT	3	8
All therapy	37	100
Median no.	2	1-8
Time from last Rx, median (mo)	7.2	0.9-67.9
First chemotherapy		
Single agent	9	25
COP or CP	11	31
Aggressive combination	17	44
Response to first chemotherapy		
CR or PR	29	81
Duration, median (mo)	12	
No response	5	14
Unknown	2	5
Response to last therapy		
CP or PR	25	86
Duration, median (mo)	12	
Unknown	2	5

* Histologic group based on Working Formulation.

mg/m^2 for 4 weeks, the highest dose used in the dose escalation phase I portion of the trial. Patient characteristics at diagnosis and prior lymphoma therapy for all 37 patients are detailed in Table 1. The median age was 58 years, with a range of 29 to 81 years. All but 1 patient had an initial diagnosis of a low-grade or follicular histology (Working Formulation [WF] groups A-D), and 76% were diagnosed with stage III or IV disease. At the time of antibody therapy, 17 patients (46%) had bone marrow involvement, 10 patients (27%) had an elevated lactate dehydrogenase (LDH) level, and 10 patients (27%) had at least one lesion greater than or equal to 7 cm. All patients had relapsed after standard chemotherapy with a median of 2 prior regimens (range, 1 to 5). Forty-four percent of patients had failed to respond to an initial aggressive combination chemotherapy regimen such as CHOP, PROMACE-CYTABOM, or MACOP-B. Fifty-six percent of patients were initially treated with a single agent (25%) or combination therapy with an alkylating agent and prednisone. Most patients (81%) had a CR or PR to initial chemotherapy with a median duration of 12 months. Relapsed disease was treated with a variety of agents including radiotherapy, single-agent, and aggressive combination chemotherapy including high-dose therapy supported with autologous stem cells (3 patients). Eighty-six percent of patients had responded to their most recent treatment with a CR (41%) or PR (45%) with a median duration of 12 months (20 patients with duration of <6 months). Treatment was administered at a median of 4.5 years from initial diagnosis (range, 0.8 to 13.3 years) and a median of 10.2 months from last therapy. Two patients had previously been treated in a phase I single-dose study using IDEC-C2B8, one of whom achieved a PR lasting 8 months.

Antibody infusions. IDEC-C2B8 infusions were administered in an outpatient setting, usually through peripheral intravenous catheters. Patients were often premedicated for the first antibody infusion with oral diphenhydramine and acetaminophen. The total dose of antibody was diluted into normal saline (generally 1 mg/mL) and administered intravenously by infusion at an initial rate of 50 mg/h, escalating every 30 minutes in the absence of side effects to a maximum of 200 mg/h.

The average dose of antibody administered during each infusion was 711 mg (range, 562 to 825 mg), with a cumulative dose over 4 infusions of 2,730 mg (maximum cumulative dose of 3,300 mg). The initial antibody infusion duration averaged 5.6 hours (range, 1.5 to 12.7 hours). In the absence of symptoms, subsequent infusions started at 200 mg/h with an average duration of infusion of 4.5, 4.4, and 4.2 hours for doses number 2, 3, and 4, respectively.

Adverse events. The most common adverse events thought to be related to treatment are detailed in Table 2. Thirty-two of the 37 patients experienced adverse events during the course of the trial; 6 patients experienced 12 grade 3 or 4 adverse events (9 reported as related to treatment). The most frequent infusion-related events included grade 1 or 2 fever (73% of patients), chills (38%), nausea (19%), asthenia (16%), headache (16%), and a sensation of tongue or throat swelling (11%). Hypotension occurred in 6 patients and was limited to grade 1 in 3 cases and grade 2 in 3 cases. Treatment-related side effects were seen most frequently with the initial antibody infusion (129 of 192 [67%]) and dramatically decreased during the second, third, and fourth infusions. Adverse events were managed by the administration of diphenhydramine or acetaminophen as indicated and by temporarily halting the antibody infusions until the symptoms abated. The infusions could in general then be restarted with minimal further toxicity. Two patients were withdrawn from study after receiving only the first of 4 planned antibody infusions (described in detail below).

Grade 3 and grade 4 adverse events included thrombocytopenia and anemia in 1 patient, abdominal pain and constipation in 1 patient, transient neutropenia at 4 months and 9 months in 2 patients, myocardial infarction in 1 patient, and malignant pleural effusion with dyspnea and traumatic hip fracture 3 weeks after antibody therapy in 1 patient. Two of the 37 patients did not complete the 4 planned antibody

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Table 2. Observed Adverse Events

	N (%)	Events (%)
Any adverse event	32 (86.5)	192 (100)
Body as a whole		
Fever	27 (73.0)	55 (28.6)
Chills	14 (37.8)	14 (7.3)
Asthenia	6 (16.2)	6 (3.1)
Headache	6 (16.2)	11 (5.7)
Cardiovascular system		
Hypotension	6 (16.2)	6 (3.1)
Digestive system		
Nausea	7 (18.9)	7 (3.6)
Vomiting	4 (10.8)	4 (2.1)
Dyspepsia	2 (5.4)	2 (1.0)
Blood and lymphatics		
Thrombocytopenia	7 (18.9)	7 (3.6)
Neutropenia	3 (8.1)	5 (2.6)
Anemia	2 (5.4)	3 (1.6)
Coagulation disorder	2 (5.4)	2 (1.0)
Leukopenia	1 (2.7)	4 (2.1)
Nervous system		
Vasodilation	1 (2.7)	3 (1.6)
Respiratory system		
Rhinitis	6 (16.2)	6 (3.1)
Laryngismus	4 (10.8)	4 (2.1)
Chest pain	2 (5.4)	2 (1.0)
Skin and appendages		
Rash	5 (13.5)	5 (2.6)
Night sweats	3 (8.1)	3 (1.6)
Urticaria	3 (8.1)	4 (2.1)
Pruritus	2 (5.4)	3 (1.6)
Special senses		
Lacrimation disorder	5 (13.5)	5 (2.6)

Included are events reported as possibly or probably related or of unknown relationship to study treatment.

Abbreviations: N, number of patients; Events, number of events.

infusions. The first patient, who had small lymphocytic lymphoma, had previously been treated with a single infusion of IDEC-C2B8 in an earlier trial and had experienced transient grade 3 thrombocytopenia. Within 24 hours of the first infusion of 375 mg/m^2 antibody, the platelet count again decreased from a baseline value of 93,000/μL to a nadir of 19,000/μL. This was associated with marked elevation in LDH level (629 to 2660 U/L), a decrease in hemoglobin level from 12.6 to 7.8 g/dL, and an elevation in uric acid to 10.5 mg/dL. She received outpatient treatment with allopurinol, prednisone, and a single platelet transfusion. Evaluation for disseminated intravascular coagulation was nondiagnostic and the patient improved (counts returned to above baseline) by day 31. The second patient, a 63-year-old man with follicular small cleaved cell NHL, had a prior history of hypertension, hypercholesterolemia, and atherosclerotic cardiovascular disease, including a prior myocardial infarction (4 months earlier); developed anginal pain within 30 minutes of initiation of his first IDEC-C2B8 infusion; and received nitroglycerin with resolution of symptoms. The antibody infusion was then completed with stable vital signs. However, the patient was hospitalized 5 days later with a myocardial infarction and was removed from study. An occluded right coronary artery was found and the patient underwent a surgical coronary artery bypass procedure.

Hematologic and chemistry laboratory effects. A total of 18 patients had some clinically significant hematologic nadir after antibody therapy and 11 of these patients had bone marrow involvement by their lymphoma. Eleven patients sustained decreases in hemoglobin or hematocrit of greater than 1.5 g or 3%, respectively. Eight patients had decreases in platelet counts to less than 100,000/μL and 10 patients had transient reductions in white blood cell count or absolute granulocyte count of less than 3,000/μL or 1,500/μL, respectively. In general, these effects were mild and patients spontaneously recovered without intervention. Two patients developed late onset isolated neutropenia of unclear etiology at 4 and 10 months after therapy. Both instances were transient and resolved within 1 month.

No clinically significant changes in chemistry laboratory values were noted.

Reported infections. A total of 9 patients (11 infections) were reported to have acquired an infection during the course of the treatment or follow-up. Infections during the first 60 days of antibody therapy included viral upper respiratory infections in 3 patients, localized skin infections in 3 patients, and herpes zoster and otitis media in 1 patient each. Reported late infections, occurring 7 to 12 months after antibody therapy, included pneumonia, impetigo, and sinusitis. All infections were grade 1 or 2 and resolved without complications after appropriate therapy.

Depletion of peripheral blood B cells. Peripheral blood lymphocytes were evaluated by flow cytometry at baseline, 72 hours after the first infusion, and at 1, 2, 6, 9, and 12 months posttreatment. The percentage and absolute number of cells expressing the CD19 and CD20 antigens was determined. Treatment with the chimeric anti-CD20 antibody rapidly and effectively depleted B cells from the peripheral blood circulation as detected by the unrelated anti-CD19 B-cell antibody. In some patients, baseline CD20 cell counts were elevated (due to the presence of circulating lymphoma cells). Most patients had a rapid decrease in the relative percentage and absolute number of CD19$^+$ cells by day 4. This level remained nearly undetectable until approximately 6 months posttreatment, followed by slow gradual recovery.

Serum Ig and complement levels. The CD20 antigen is expressed on early B cells but not upon differentiation to antibody secreting plasma cell. Because of the possibility that the elimination of normal B cells and plasma cell progenitors could affect antibody production, serum Ig (IgG, IgA, and IgM) levels were quantitated at baseline and throughout the study. Mean serum Ig levels remained stable; however, individual patients experienced transient decreases in some subsets. Among these 37 patients, 4, 4, and 5 patients experienced more than a 20% decline in IgG, IgA, or IgM levels, respectively (total of 10 patients with 3 patients having decreases in both IgG and IgA levels).

No clinically significant change in serum complement levels was observed.

Evaluation of immune response (HAMA/HACA). Of the 37 patients evaluated, there were no quantifiable HAMA or HACA titers identified. One patient (with a PR) had a

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Table 3. Summary of Clinical Response

Patient Group	N	CR (%)	PR (%)	CR and PR (%)	95% CI (CR and PR)
Intent to treat	37	3 (8)	14 (38)	17 (46)	30-62%
Evaluable*	34	3 (9)	14 (41)	17 (50)	33-67%

Abbreviation: CI, confidence interval.

* Patients had low-grade or follicular lymphoma and completed four antibody infusions.

detectable, but not a quantifiable (<100 ng/mL) HACA titer 7 months posttreatment. There was no toxicity related to this immune response.

Clinical response. Clinical responses (CR or PR) were observed in 17 of the 37 patients (46%) and are detailed in Table 3. Two patients were withdrawn from the study for adverse events after receiving a single infusion of the antibody and 1 patient had a high-grade B-cell malignancy. Thus, 34 patients completed all four antibody infusions and were considered evaluable for response. All 34 had a low-grade or follicular histology. Three of the 34 patients (9%) had a CR and 14 of 34 (41%) had a PR for an overall response rate for evaluable patients of 50%. An additional 8 patients had some evidence of tumor response (>20% reduction), but did not qualify as responders (PR or CR). Thus, 25 of the 34 evaluable patients (74%) had evidence of antitumor effect. Eleven patients (32%) had stable disease (SD) and 6 patients (18%) were judged to have progressive disease (PD). The median time to onset of clinical response for the 17 responders was 50 days (range, 7 to 112 days). Measured tumor masses continued to regress, with the maximum response in sentinel tumor lesions occurring 3 to 4 months after antibody therapy. The median time to progression for the clinical responders was 10.2 months, with 5 patients exceeding 20 months and 2 patients exceeding 30 months. The median duration of response was 8.6 months. A Kaplan-Meier survival curve for the time to progression is shown in Fig 1 for the clinical responders.

The majority of the patients had follicular histologies. Of 21 patients with follicular small cleaved histology (WF group B), there were 11 PR and 2 CR for a response rate of 62%. Of 9 patients with follicular mixed small and large cell histology (WF group C), there was 1 CR and 3 PR (44%). One of 2 patients with follicular large cell lymphoma (group D) had a PR. The response rate appears similar among the follicular histologies. In contrast, none of 4 patients with small lymphocytic lymphoma (1 with mantle cell histology) had a clinical remission.

There was no clear influence of type of prior therapy on the likelihood of clinical response to antibody treatment. Two of three patients who had been treated previously with high-dose therapy and autologous stem cell rescue achieved a PR to antibody therapy with times to disease progression of 4.2 and 21.7 months. Twenty patients had extranodal disease and two showed a CR and seven showed a PR to antibody therapy. There was a correlation observed between the duration of response to last chemotherapy and response to treatment with IDEC-C2B8 ($P = .05$; Fisher's exact test). Twenty of the 37 patients had a duration of response to last chemotherapy of 6 months or less. Of these 20, 6 (30%) responded to chimeric MoAb therapy. Seventeen of the 37 patients had a duration of response to last chemotherapy of more than 6 months. Of these 17, 11 (65%) responded to IDEC-C2B8.

Antibody pharmacokinetics. At the 375 mg/m^2 dose used in this trial, all studied patients exhibited detectable circulating antibody throughout the treatment period. Preinfusion (doses 2 through 4) antibody concentrations ranged from 0.8 to 517.7 μg/mL and postinfusion levels ranged from 107.8 to 962.7 μg/mL. Five patients consistently had preinfusion antibody levels at or less than 10 μg/mL, suggesting that they did not reach steady state or cleared the antibody at a faster rate. Most patients exhibited increasing preinfusion antibody concentrations with each subsequent infusion.

The Wilcoxon rank sum test was used to examine the relationship between the preinfusion and postinfusion chime-

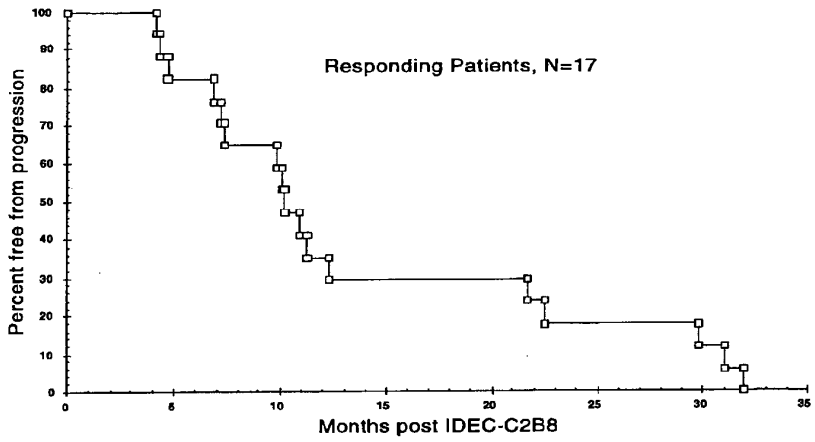


Fig 1. Kaplan-Meier graph of the time to progression from the date of initial treatment with the first dose of IDEC-C2B8 until documented tumor relapse or progression in the 17 clinical responders.

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ric antibody concentrations and the clinical response. A correlation was observed between clinical response and the median values of IDEC-C2B8 serum levels before the second infusion ($P = .029$), with responders having a median of 82.7 μg/mL (range, 1.2 to 125.3 μg/mL) versus nonresponders with a median of 21.9 μg/mL (range, 1.1 to 99.0 μg/mL). Detailed pharmacokinetic sampling was performed on 9 patients, with a mean C_{max} of 500.5 ± 135.2 μg/mL (range, 201.4 to 663.0 μg/mL) and serum antibody half-life values from 12.7 to 370.8 hours (mean $T_{1/2}$ of 225.9 ± 102.7 hours).

DISCUSSION

IDEC-C2B8 represents a new treatment for patients with recurrent low-grade or follicular NHL. This therapy has a response rate in evaluable patients of 50% and a time to progression of 10.2 months. The treatment consists of four intravenous infusions administered over a 22-day period in the outpatient setting with minimal toxicity. This compares favorably with other agents currently in use for the treatment of patients with relapsed disease. These results have recently been confirmed in preliminary reports in a separate phase II/III multicenter trial in more than 150 patients.[21]

The adverse events observed in this trial were predominately infusion related and generally consisted of mild grade 1 or 2 symptoms of fever, chills, rigor, rash, nausea, and, rarely, mild respiratory symptoms or hypotension. All of these side effects were transient and did not interfere with the completion of the planned antibody infusion. Two patients were removed from study after a single dose of the antibody; 1 patient developed thrombocytopenia and an elevated LDH and a second patient with known cardiac disease experienced chest pain and was admitted 1 week later with a myocardial infarction. All other patients completed the planned 4 weekly infusions. The etiology of the observed infusion related symptoms is not completely understood, but may represent the rapid depletion of B cells and lysis in the reticuloendothelial systems of the lung, liver, and spleen. This constellation of symptoms has been seen in other studies using murine MoAbs.[22] The marked decrease in infusional side effects noted with subsequent infusions likely reflects the elimination of most circulating B lymphocytes, the saturation of antigenic sites with the first infusion, and persistent antibody serum levels present at the time of the subsequent antibody infusions.

As expected, normal B cells were rapidly depleted from the peripheral blood of nearly all patients and remained depleted until nearly 6 months posttreatment, followed by a slow recovery. Despite this depletion of B cells, there was minimal change in serum Ig levels and no increase in the frequency or severity of infectious complications. The infections noted in this trial are common to patients with relapsed B-cell lymphoma and are not clearly related to treatment with the antibody. Prolonged B-cell depletion is common to other chemotherapy treatments and after BMT. The type and risk of infection with MoAb treatment appears less than that reported with administration of some of the newer purine analogues (fludarabine and 2-CDA) that are associated with significant depletion of the $CD4^+$ T-cell population.

The patients included in this trial are typical of those represented in the general population and of low-grade patients studied in the Working Formulation Lymphoma Classification Project.[23] The International Prognostic Index (IPI) originally developed for aggressive NHL uses 5 prognostic factors, including age greater than 60 years; stage III/IV disease; performance status greater than 1; elevated LDH level; and extranodal sites greater than 1.[24] It has been applied to patients with low-grade NHL and permits prognostic stratification into risk groups.[25] Using data from presentation, we were able to determine the IPI classification in 32 of the 37 patients included in this trial. As expected, in patients with low-grade NHL, the majority of patients were in the low (41%) and low-intermediate (27%) prognostic groups, followed by those in intermediate/high (16%), high (3%), and unknown (14%) groups. There was no statistically significant correlation (Fisher's exact test, $P = .31$) between the risk groups and response to treatment with IDEC-C2B8. However, there was a correlation observed between the duration of response to last chemotherapy and the response to IDEC-C2B8. Patients with less than a 6-month response to last chemotherapy had a 30% response rate, compared with a 65% response rate for patients with last duration of remission of greater than 6 months. In contrast with the experience with some MoAbs, antitumor activity was observed in patients with bulky disease and in poor prognostic patients who had relapsed after anthracycline-containing regimens or after high-dose therapy with stem cell support. Tumor responses were noted in patients with bulky lymph nodes, splenomegaly, extranodal masses, and lymphoma in blood and bone marrow.

A correlation was also observed between the level of IDEC-C2B8 present in the serum before infusion number 2 and the response to MoAb therapy. Whether this is due to differences in tumor burden or to differences in intrinsic clearance of the MoAb is unknown. All 17 clinical remissions occurred in patients with a follicular histology (WF groups B-D), although the number of patients with other histologies was small. In contrast, none of the 4 patients with small lymphocytic lymphoma (WF group A) responded. These patients had heterogenous disease, including 1 patient with mantle cell histology. Although patients with chronic lymphocytic leukemia (CLL) were excluded from this trial (based on the presence of >5,000 lymphocytes/μL for this histologic subgroup), it is possible that the decreased response rate in this subgroup was due to a lower expression of the CD20 surface antigen that has been observed in cases of CLL.

The method of tumor cell killing by the MoAb is not completely understood and likely involves several mechanisms. The chimeric MoAb was engineered to contain human IgG1 constant regions to augment immune-mediated antitumor activity using human complement and human ADCC effector cells.[16] In vitro, the antibody clearly exhibits much greater activity than the parent murine IgG1 MoAb in these assays. However, in this clinical trial, there were no significant changes in serum complement levels observed after the MoAb treatments. In addition, in earlier phase I trials, tumor biopsies 2 weeks posttreatment showed antibody bound to tumor cells in patients thought to be immunocompetent who

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did not have a clinical remission.[18] The CD20 molecule is thought to aggregate on the cell surface and create or control a Ca^{2+} channel that is important in initiation and progression through the cell cycle.[26] Antibodies binding to CD20 may directly inhibit the cell cycle.[27] Recent data using IDEC-C2B8 have shown that the antibody inhibits proliferation and directly induces apoptosis in some B-cell NHL lines in vitro.[17] The degree to which this additional antitumor mechanism contributes to the observed clinical remissions is unknown and is actively being evaluated in the laboratory.

The optimal treatment for patients with advanced-stage or relapsed low-grade NHL remains unsettled. Diverse options, from watch and wait to treatment with aggressive chemotherapy, are frequently used.[1] Although most patients initially respond to a variety of chemotherapeutic regimens with a PR or CR, all will eventually relapse and require additional treatment with decreasing response rates and decreasing durations of remission. Aggressive combination chemotherapy produces higher CR rates, and high-dose therapy with stem cell or bone marrow support may result in longer durations of remission. Unfortunately, most trials of such aggressive therapies have shown continued risk of relapse without a convincing plateau on the failure-free survival curve.[2] An exception may be allogenic BMT, but this treatment is restricted to younger patients with an appropriate donor and carries a high risk of morbidity and mortality.[28] Most patients undergo a series of treatments with increasing toxicity and decreasing bone marrow reserve until they ultimately become refractory to treatment and die of disease progression or from complications of treatment.

IDEC-C2B8 chimeric anti-CD20 MoAb presents the opportunity to obtain meaningful tumor reductions with minimal toxicity in patients with relapsed low-grade NHL. Retreatment is possible and early experience suggests that a majority of patients with a prior response may respond to retreatment. Fludarabine has also been studied in patients with relapsed disease, with an overall response rate of approximately 40%.[29,30] Time to treatment failure was 4.6 months in one study.[31] Treatment complications from fludarabine included infections or fever of unknown origin in 62% of patients, bone marrow suppression, and opportunistic infections. Similar responses have been observed with cladribine; however, marked immunosuppression and hematologic toxicity was noted.[32,33]

To date, experience using IDEC-C2B8 has been predominately in patients with relapsed measurable disease. A clinical trial combining IDEC-C2B8 with 6 cycles of CHOP chemotherapy in newly diagnosed patients has recently been completed. Early evaluation of this experience suggests that this combination resulted in a PR or CR in all patients and in the clearance of polymerase chain reaction-detectable cells from the peripheral blood and bone marrow in 7 of 8 patients with a positive pretreatment assay.[34] It remains to be determined if this will ultimately decrease the risk of relapse or prolong disease-free survival. Additional areas that should be investigated using this new agent include (1) extended and repeated dosing regimens, (2) combination with or after standard chemotherapy, (3) use as an in vivo B-cell purging agent before collection of bone marrow or stem cells for high-dose therapies, (4) evaluation in other B-cell histologies, and (5) lymphomas arising in association with immunodeficiency such as acquired immunodeficiency syndrome or organ transplantation.

In conclusion, this experience using an engineered chimeric MoAb finally provides practical substance to the promise of antibody therapy of cancer that was envisioned nearly 100 years ago by Ehrlich.[35]

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IDEC-C2B8 (Rituximab) Anti-CD20 Monoclonal Antibody Therapy in Patients With Relapsed Low-Grade Non-Hodgkin's Lymphoma

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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.).

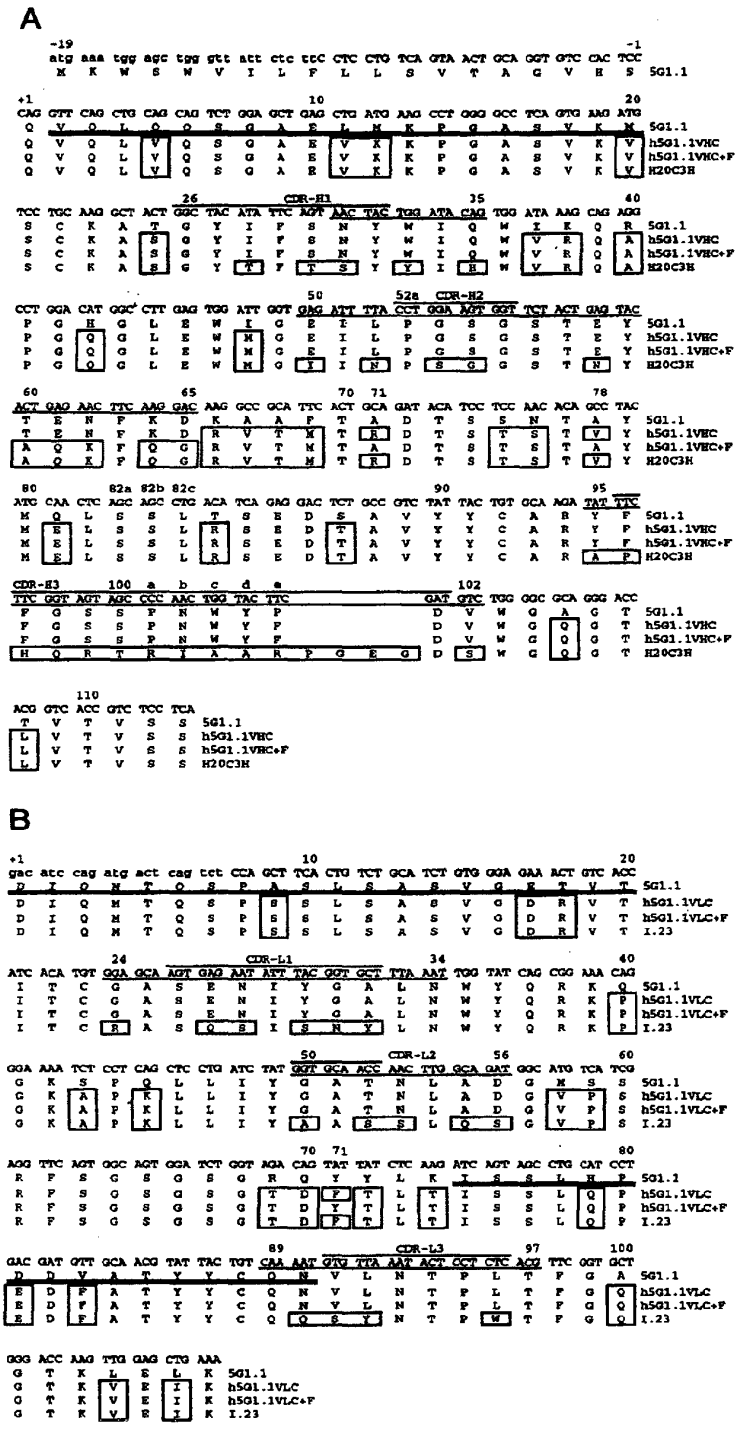
Construction of a humanized scFv

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

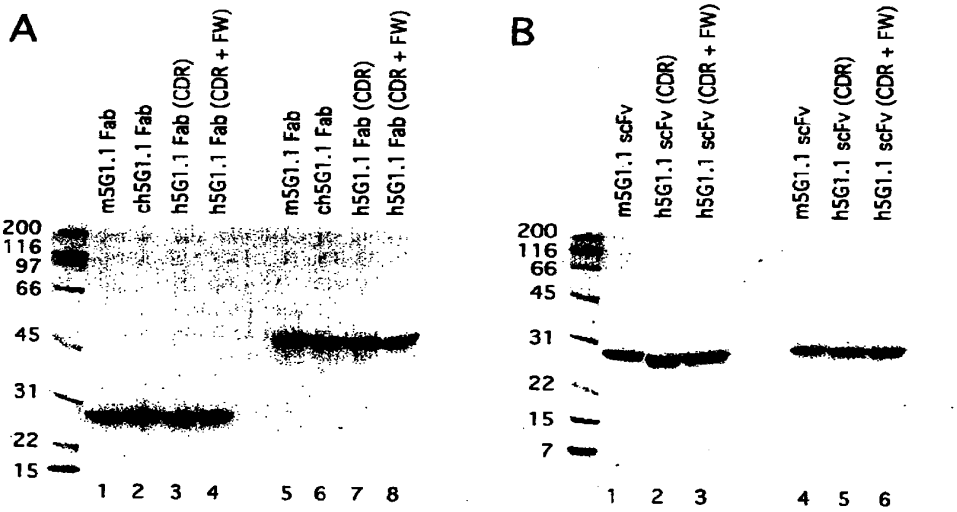


Fig. 2. SDS–PAGE analysis of purified recombinant proteins. (A) Fab fragments from the murine 5G1.1 mAb (m5G1.1 Fab) were produced by papain digestion of the m5G1.1 mAb followed by protein A chromatography to remove undigested antibody and Fc fragments. Recombinant chimeric Fab (ch5G1.1 Fab), CDR-grafted 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.

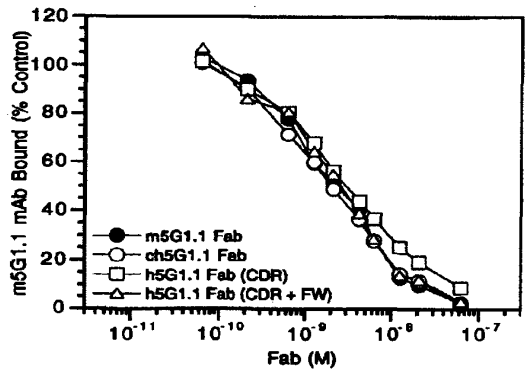


Fig. 3. Inhibition of 5G1.1 mAb binding to human C5 by recombinant Fab. 5G1.1 (0.2 nM) mAb plus the indicated concentrations of recombinant Fab were incubated together, for 2 hr at 37°C in microtiter plates coated with human C5. After washing, bound 5G1.1 mAb was detected using peroxidase-conjugated 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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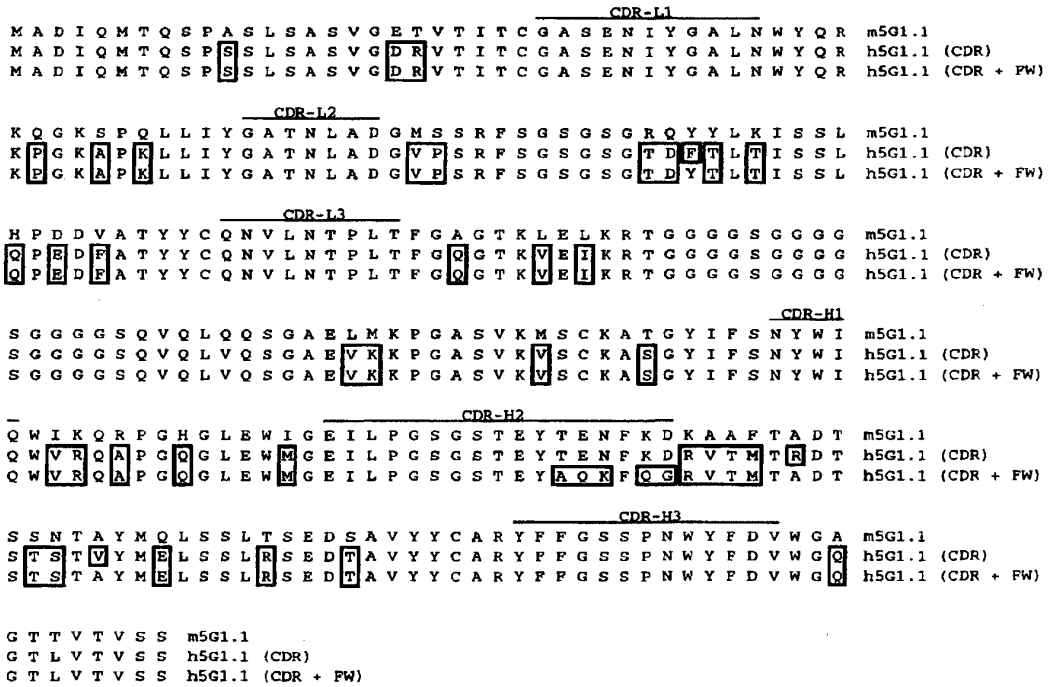


Fig. 4. Sequence of scFv molecules constructed using the 5G1.1 murine variable regions (m5G1.1) or humanized variable regions. The h5G1.1 (CDR) scFv contains the variable regions h5G1.1 VLC and h5G1.1 VHC, while the h5G1.1 scFv (CDR + FW) contains the variable regions h5G1.1 VLC+F and h5G1.1 VHC+F. The 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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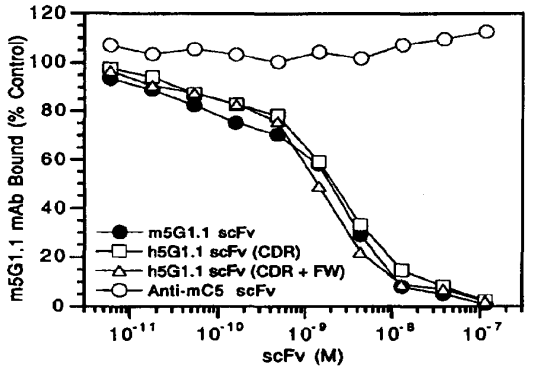


Fig. 5. Inhibition of 5G1.1 mAb binding to human C5 by recombinant scFv. 5G1.1 mAb (0.2 nM) plus the indicated concentrations of recombinant scFv were incubated together for 2 hr at 37°C, in microtiter plates coated directly with human C5. After washing, bound 5G1.1 mAb was detected using peroxidase-conjugated 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.

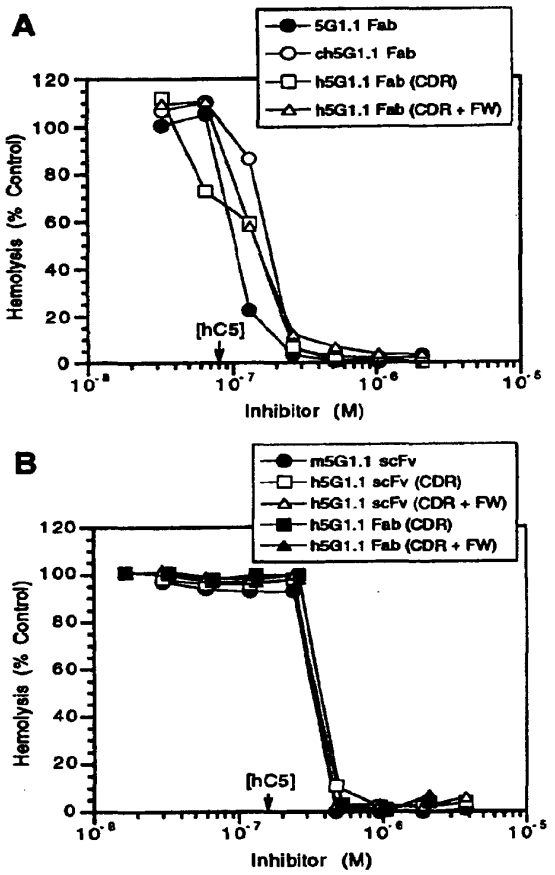


Fig. 6. Inhibition of chicken erythrocyte lysis by recombinant Fab and scFv. Twenty percent (A), or 40% (B), human serum in $GVBS^{++}$ was pre-incubated for 30 min at room temperature, with the indicated concentrations of competitors. Chicken erythrocytes, precoated with rabbit anti-chicken erythrocyte 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

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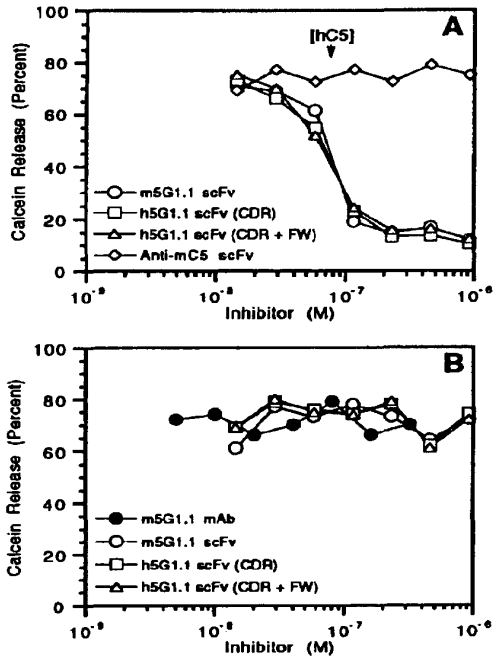


Fig. 7. Protection of porcine aortic endothelial cells (PAEC) from human serum complement by scFv. Calcein-labeled PAEC were incubated with HBSS containing 1% BSA and 0.85 mg/ml purified rabbit anti-PAEC polyclonal antibody for 30 min at 37°C. The cells were washed and incubated with HBSS containing 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.

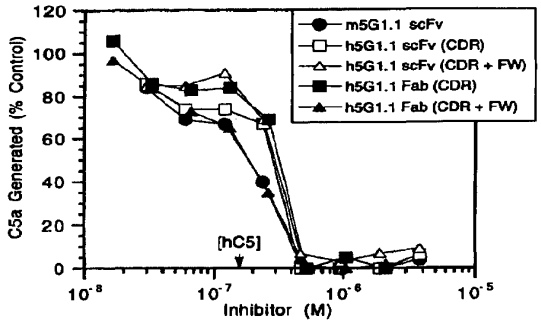


Fig. 8. Inhibition of C5a generation by recombinant Fab and scFv. Supernatants from chicken erythrocyte hemolytic assays performed using 40% human serum, as described in Fig. 5, were assayed for C5a content by ELISA. The C5a concentration obtained in the absence of inhibitor was defined as 100%. Data shown are from a representative experiment of several performed. The predicted hC5 concentration in 40% serum is indicated by the arrow.

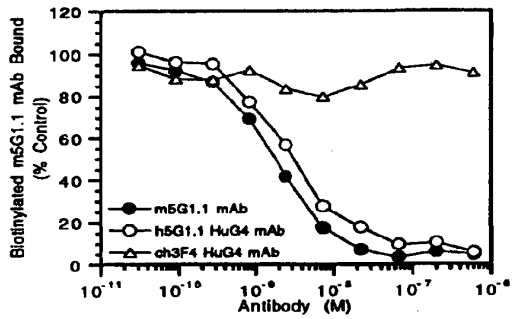


Fig. 9. Inhibition of 5G1.1 mAb binding to human C5 by recombinant humanized h5G1.1 HuG4 mAb. Biotinylated 5G1.1 mAb (0.2 nM), plus the indicated concentrations of unlabeled murine 5G1.1 mAb, recombinant h5G1.1 HuG4 mAb or a negative control recombinant HuG4 mAb (ch3F4 HuG4 mAb), were incubated together 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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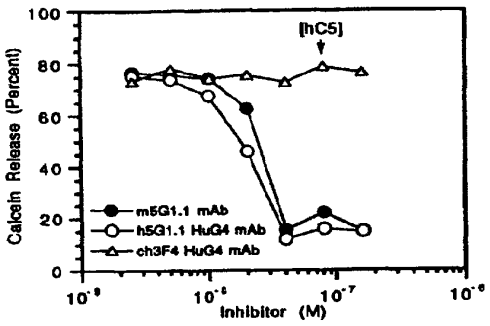


Fig. 10. Protection of porcine aortic endothelial cells from human serum complement by h5G1.1 HuG4 mAb. Calcein-labeled PAEC were incubated with HBSS containing 1% BSA and 0.85 mg/ml purified rabbit anti-PAEC polyclonal antibody for 30 min at 37°C. The cells were washed and incubated with HBSS containing 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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ANNEX II: Commercial monoclonal antibodies 2006 (for intravenous administration)

Antibody	Trade name	Year	Concentration [mg/ml] *	Concentration range [mg/ml]			
				0.1 - 50	0.5 - 25	2 - 10	10
Gemtuzumab ozogamicin	Mylotarg	2000	0.25	X			
Indium-111 capromab pendetide	ProstaScint	1996	0.5	X	X		
Satumomab pendetide	OncoScint	1992	0.5	X	X		
Muromomab-CD3	Orthoclone OKT	1986	1	X	X		
Acrituomab; Tc-99	CEA-Scan	1996	1.25	X	X		
Ibritumomab-Tiuxetan	Zevalin		1.6	X	X		
Abciximab	ReoPro	1994	2	X	X	X	
Cetuximab	Erbitux	2004	2	X	X	X	
Basiliximab	Simulect	1998	4	X	X	X	
Daclizumab	Zenapax	1997	5	X	X	X	
Alemtuzumab	Campath	2001	10	X	X	X	X
Infliximab	Remicade	1998	10	X	X	X	X
Nofetumomab	Verluma	1996	10	X	X	X	X
Rituximab	Rituxan	1997	10	X	X	X	X
Tositumomab and I-131 Tositumomab	Bexxar	2003	14	X	X		
Natalizumab	Tysabri	2004	20	X	X		
Trastuzumab	Herceptin	1998	21	X	X		
Bevacizumab	Avastin	2004	25	X	X		
Number of antibodies (total number: 18)				**18**	**17**	**8**	**4**
Proportion of all antibodies				**100 %**	**94 %**	**44 %**	**22 %**

* Liquid form or after reconstitution

Exhibit no. ANNEX II
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ANNEX I: Commercial monoclonal antibodies 2006 (all modes of administration)

Antibody	Trade name	Year	Concentration [mg/ml] *	Concentration range [mg/ml]			
				0.1 - 50	0.5 - 25	2 - 10	10
Gemtuzumab ozogamicin	Mylotarg	2000	0.25	X			
Indium-111 capromab pendetide	ProstaScint	1996	0.5	X	X		
Satumomab pendetide	OncoScint	1992	0.5	X	X		
Muromomab-CD3	Orthoclone OKT	1986	1	X	X		
Acrituomab; Tc-99	CEA-Scan	1996	1.25	X	X		
Ibritumomab-Tiuxetan	Zevalin		1.6	X	X		
Abciximab	ReoPro	1994	2	X	X	X	
Cetuximab	Erbitux	2004	2	X	X	X	
Basiliximab	Simulect	1998	4	X	X	X	
Daclizumab	Zenapax	1997	5	X	X	X	
Alemtuzumab	Campath	2001	10	X	X	X	X
Infliximab	Remicade	1998	10	X	X	X	X
Nofetumomab	Verluma	1996	10	X	X	X	X
Ranibizumab	Lucentis	2006	10	X	X	X	X
Rituximab	Rituxan	1997	10	X	X	X	X
Tositumomab and I-131 Tositumomab	Bexxar	2003	14	X	X		
Natalizumab	Tysabri	2004	20	X	X		
Trastuzumab	Herceptin	1998	21	X	X		
Bevacizumab	Avastin	2004	25	X	X		
Adalimumab	Humira	2002	50	X			
Efalizumab	Raptiva	2003	100				
Palivizumab	Synagis	1998	100				
Omalizumab	Xolair		125				
Number of antibodies (total number: 23)				**20**	**18**	**9**	**5**
Proportion of all antibodies				**87 %**	**78 %**	**39 %**	**22 %**

* Liquid form or after reconstitution

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MINIREVIEW

Antibody Structure, Instability, and Formulation

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ABSTRACT: The number of therapeutic monoclonal antibody in development has increased tremendously over the last several years and this trend continues. At present there are more than 23 approved antibodies on the US market and an estimated 200 or more are in development. Although antibodies share certain structural similarities, development of commercially viable antibody pharmaceuticals has not been straightforward because of their unique and somewhat unpredictable solution behavior. This article reviews the structure and function of antibodies and the mechanisms of physical and chemical instabilities. Various aspects of formulation development have been examined to identify the critical attributes for the stabilization of antibodies. © 2006 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 96:1–26, 2007

Keywords: biotechnology; stabilization; protein formulation; protein aggregation; freeze drying/lyophilization

INTRODUCTION

Protein therapies are entering a new era with the influx of a significant number of antibody pharmaceuticals. Generally, protein drugs are effective at low concentrations with less side effects relative to small molecule drugs, even though, in rare cases, protein-induced antibody formation could be serious.[1] Therefore, this category of therapeutics is gaining tremendous momentum and widespread recognition both in small and large drug firms. Among protein drug therapies, antibodies play a major role in controlling many types of diseases such as cancer, infectious diseases, allergy, autoimmune diseases, and inflammation. Since the approval of the first monoclonal antibody (MAb) product -OKT-3 in 1986, more than 23 MAb drug products have entered the market (Tab. 1). The estimated number of antibodies and antibody derivatives constitute 20% of biopharmaceutical products currently in development (about 200).[2] The global therapeutic antibody market was predicted to reach $16.7 billion in 2008.[3]

There are several reasons for the increasing popularity of antibodies for commercial development. First, their action is specific, generally leading to fewer side effects. Second, antibodies may be conjugated to another therapeutic entity for efficient delivery of this entity to a target site, thus reducing potential side effects. For instance, Mylotarg is an approved chemotherapy agent composed of calicheamicin conjugated to humanized IgG4, which binds specifically to CD33 for the treatment of CD33-positive acute myeloid leukemia. Another example is the conjugation of immunotoxic barnase with the light chain of the anti-human ferritin monoclonal antibody F11 as potential targeting agents for cancer immunotherapy.[4] Third, antibodies may be conjugated to radioisotopes for specific diagnostic purposes. Examples include CEA-Scan for detection of colorectal cancer and ProstaScint for detection of prostate cancer. Lastly, technology advancement has made complete human MAb available, which are less immunogenic.

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Table 1. Commercial Monoclonal Antibody Products

#	Brand name	Molecule	MAb	Year	Company	Route	Indication	MAb Conc	Buffer	Excipients	Surfactant	pH
1	Avastin	Bevacizumab	Humanized IgG1, 149 kDa	2004	Genetech and BioOncology	IV infusion	Metastatic carcinoma of colon or rectum, binds VEGF	100 mg and 400 mg/vial (25 mg/mL) solution	5.8 mg/mL monobasic NaPhos H_2O; 1.2 mg/mL dibasic NaPhos anhydrous (4 mL, 16 mL fill in vial)	60 mg/mL α-Trehalose dihydrate (4 mL, 16 mL fill in vial)	0.4 mg/mL PS20 (4 mL, 16 mL fill in vial)	6.2
2	Bexxar	Tositumomab and I-131Tositumab	Murine IgG2λ	2003	Corixa and GSK	IV Infusion	CD20 positive follicular nonHodgkins lymphoma	Kit: 14 mg/mL MAb solution in 35 mg and 225 mg vials; 1.1 mg/mL *I131-MAb* solution	10 mM phosphate (MAb vial)	145 mM NaCl, 10 % w/v Maltose; *I131-MAb*: 5–6% Povidone, 1–2, 9–15 mg/mL Maltose, 0.9 mg/mL NaCl, 0.9–1.3 mg/mL Ascorbic acid		7.2
3	Campath	Alemtuzumab	Humanized, IgG1k, 150 kDa	2001	Ilex Oncology; Millenium and Berlex	IV infusion	B-cell chronic lymphocytic leukemia, CD52-antigen	30 mg/3 mL solution	3.5 mg/3 mL dibasic NaPhos, 0.6 mg/3 mL monobasic KPhos	24 mg/3 mL NaCl, 0.6 mg/3 mL KCl, 0.056 mg/3 mL Na2EDTA	0.3 mg/3 mL PS80	6.8–7.4
4	CEA-Scan (lyo)	Arcitumomab; Tc-99	Murine Fab, 50 kDa	1996	Immunomedics	IV injection or infusion	Imaging agent for colorectal cancer	1.25 mg/vial Lyophilized MAb. Reconstitute w 1 mL Saline w Tc99m	0.29 mg/vial Stannous chloride, potassium sodium tartrate tetrahydrate, NaAcetate.$3H_2O$, NaCl, glacial acetic acid, HCl	Sucrose		5.7
5	Erbitux	Cetuximab	Chimeric human/ mouse IgG1k, 152 kDa	2004	ImClone and BMS	IV infusion	Treatment of EGFR-expressing colorectal carcinoma	100 mg MAb in 50 mL; 2 mg/ mL solution	1.88 mg/mL DibasicNaPhos · $7H_2O$; 0.42 mg/mL MonobasicNaPhos · H_2O	8.48 mg/mL NaCl		7.0–7.4
6	Herceptin (lyo)	Trastuzumab	Humanized IgG1k	1998	Genetech	IV infusion	Metastatic breast cancer whose tumor overexpress HER2 protein	440 mg /vial, 21 mg/mL after reconstitution	9.9 mg/20 mL L-HistidineHCl, 6.4 mg/20 mL L-Histidine	400 mg/20 mL α-Trehalose Dihydrate	1.8 mg/20 mL PS20	6
7	Humira	Adalimumab	Human IgG1k, 148 kDa	2002	CAT and Abbott	SC	RA patients not responding to DMARDs. Blocks TNF-alpha	40 mg/0.8 mL solution (50 mg/mL)	0.69 mg/0.8 mL Monobasic NaPhos · $2H_2O$; 1.22 mg/0.8 mL DibasicNaPhos · $2H_2O$; 0.24 mg/0.8 mL NaCitrate, 1.04 mg/ 0.8 mL Citric acid · H_2O	4.93 mg/0.8 mL NaCl; 9.6 mg/0.8 mL Mannnitol	0.8 mg/0.8 mL PS80	5.2
8	Lucentis	Ranibizumab	Humanized IgG1k fragment	2006	Genentech	Intravitreal injection	Age-related macular degeneration (wet)	10 mg/mL solution	10 mM HistidineHCl	10% α-Trehalose-Dihydrate	0.01% PS20	5.5

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9	Mylotarg (lyo)	Gemtuzumab ozogamicin	Humanized IgG4k conjugated with calicheamicin	2000	Celltech and Wyeth	IV infusion	Humenized Ab linked to calicheamicin for treatment of CD33 positive acute myeloid leukemia	5 mg protein-equivalent lyophilized powder/20-mL vial	Monobasic and dibasic NaPhosphate	Dextran 40, Sucrose, NaCl		
10	OncoScint	Satumomab pendetide	Murine IgG1k conjugated to GYK-DTPA	1992	Cytogen	IV injection	Imaging agent for colorectal and ovarian cancer	0.5 mg conjugate/mL solution (2 mL per vial)	Phosphate buffer saline			6.0
11	Orthoclone OKT	Muromomab-CD3	Murine, IgG2a, 170 kDa	1986	Ortho Biotech	IV injection	Reversal of acute kidney transplant rejection (anti CD3-antigen)	1 mg/mL solution	2.25 mg/5 mL monobasic NaPhos, 9.0 mg/5 mL dibasic NaPhos	43 mg/5 mL NaCl	1 mg/mL PS80	7 ± 0.5
12	ProstaScint	Indium-111 capromab pendetide	Murine IgG1k-conjugated to GYK-DTPA	1996	Cytogen	IV injection	Imaging agent for prostate cancer	0.5 mg conjugate/mL solution (1 mL per vial)	Phosphate buffer saline			5–7
13	Raptiva (lyo)	Efalizumab	Humanized IgG1k	2003	Xoma and Genentech	SC	Chronic moderate to severe plaque psoriasis, binds to CD11a subunit of LFA-1	150 mg MAb/vial; 125 mg/ 1.25 mL (100 mg/mL) after reconstitution with 1.3 mL SWFI	6.8 mg/vial L-HistidineHCl·H_2O; 4.3 mg/vial L-IIistidine	123.2 mg/vial Sucrose	3 mg/vial PS20	6.2
14	Remicade (lyo)	Infliximab	Chimeric human/ murine MAb against TNFalpha (app. 30% murine, 70% corresponds to human IgG1 heavy chain and human kappa light chain constant regions)	1998	Centocor	IV infusion	RA and Crohn's disease (anti TNFalpha)	100 mg / 20-mL Vial, 10 mg/mL on reconstitution	2.2 mg/10 mL MonobasicNaPhos H_2O, 6.1 mg/10 mL DibasicNaPhos·$2H_2O$	500 mg/10 mL Sucrose	0.5 mg/10 mL PS80	7.2
15	ReoPro	Abciximab	Fab. Chimeric human-murine, 48 kDa	1994	Centocor / Lilly	IV injection and infusion	Reduction of acute blood clot related complications	2 mg/mL solution	0.01 M NaPhosphate	0.15 M NaCl	0.001% (0.01 mg/ mL) PS80	7.2
16	Rituxan	Rituximab	Chimeric mouse/ human IgG1k with murine light and heavy chain variable region (Fab domain), 145 kDa	1997	IDEC and Genentech	IV infusion	NonHodgkin's lymphoma. (anti CD20-antigen)	10 mg/mL solution	7.35 mg/mL NaCitrate·$2H_2O$	9 mg/mL NaCl	0.7 mg/mL PS80	6.5

(Continued)

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Table 1. *(Continued)*

#	Brand name	Molecule	MAb	Year	Company	Route	Indication	MAb Conc	Buffer	Excipients	Surfactant	pH
17	Simulect (lyo)	Basiliximab	Chimaric IgG1k, 144 kDa	1998	Novartis	IV injection and infusion	Prevention of acute kidney transplant rejection, IL-2 receptor antagonist	10 mg and 20 mg/ vial, 4 mg/mL on reconstitution	3.61 mg, 7.21 mg Monobasic KPhos; 0.50 mg, 0.99 mg Na_2HPO_4	0.8 mg, 1.61 mg NaCl; 10 mg, 20 mg Sucrose; 40 mg, 80 mg Mannitol; 20 mg 40 mg Glycine		
18	Synagis (lyo)	Palivizumab	Humanized IgG1k, CDR of murine MAb 1129, 148 kDa	1998	MedImmune	IM injection	Prevent replication of the Respiratory syncytial virus (RSV)	50 mg and 100 mg / vial, 100 mg/mL on reconstitution	47 mM Histidine, 3.0 mM Glycine	5.6 % Mannitol		
19	Tysabri	Natalizumab	Humainzed IgG4k	2004	Biogen IDEC	IV Infusion	MS relapse	300 mg/15 mL solution	17.0 mg MonobasicNa Phos·H_2O, 7.24 mg diBasicNaPhos·$7H_2O$ for 15 mL	123 mg/15 mL NaCl	3.0 mg/15 mL PS80	6.1
20	Verluma	Nofetumomab	Murine Fab	1996	Boehringer Ingelheim and DuPont Merck	IV injection	Imaging agent for lung cancer	10 mg/mL solution	Phosphate buffer saline			?
21	Xolair (lyo)	Omalizumab	Humanized IgG1k, 149 kDa		Genentech w Novartis and Tanox	SC	Asthma, inhibits binding of IgE to IgE receptor FCeRI	202.5 mg/vial, Deliver 150 mg/ 1.2 mL on reconstitution with 1.4 mL SWFI	2.8 mg LHistidineHCl·H_2O; 1.8 mg LHistidine	145.5 mg Sucrose	0.5 mg PS20	
22	Zenapax	Daclizumab	Humanized IgG1, 144 kDa	1997	Roche	IV infusion	Prophylaxis of acute organ rejection in patients receiving renal transplants. Inhibits IL-2 binding to the Tac subunit of IL-2 receptor complex	25 mg/5 mL MAb Solution	3.6 mg/mL MonobasicNa Phos·H_2O; 11 mg/mL DibasicNaPhos·$7H_2O$	4.6 mg/mL NaCl	0.2 mg/mL PS80	6.9
23	Zevalin	Ibritumomab-Tiuxetan	Murine IgG1k-thiourea covalent linkage to Tiuxetan		IDEC	IV infusion	CD20 antigen. (Kit with Ytterium-90 induces cellular damage by beta emission)	3.2 mg/2 mL solution		09% NaCl		7.1

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Development of commercially viable antibody pharmaceuticals has, however, not been straightforward. This is because the behavior of antibodies seems to vary, even though they have similar structures. In attempting to address some of the challenges in developing antibody therapeutics, Harris et al.[5] reviewed the commercial-scale formulation and characterization of therapeutic recombinant antibodies. In a different review, antibody production and purification have been discussed.[2] Nevertheless, the overall instability and stabilization of antibody drug candidates have not been carefully examined in the literature. This article, not meant to be exhaustive, intends to review the structure and functions of antibodies, discuss their instabilities, and summarize the methods for stabilizing/formulating antibodies.

ANTIBODY STRUCTURE

Antibodies (immunoglobulins) are roughly Y-shaped molecules or combination of such molecules (Fig. 1). Their structures are divided into two regions—the variable (V) region (top of the Y) defining antigen-binding properties and the constant (C) region (stem of the Y), interacting with effector cells and molecules. Immunoglobulins can be divided into five different classes –IgA, IgD, IgE, IgM, and IgG based on their C regions, respectively designated as α, δ, ε, μ, and γ (five main heavy-chain classes).[6] Most IgGs are monomers, but IgA and IgM are respectively, dimmers and pentamers linked by J chains. IgGs are the most abundant, widely used for therapeutic purposes, and their structures will be discussed as antibody examples in detail.

Primary Structure

The structure of IgGs have been thoroughly reviewed.[6] The features of the primary structure of antibodies include heavy and light chains, glycosylation, disulfide bond, and heterogeneity.

Heavy and Light Chains

IgGs contain two identical heavy (H, 50 kDa) and two identical light (L, 25 kDa) chains (Fig. 1). Therefore, the total molecular weight is approximately 150 kDa. There are several disulfide bonds linking the two heavy chains, linking the heavy and light chains, and residing inside the chains (also see next section). IgGs are further divided

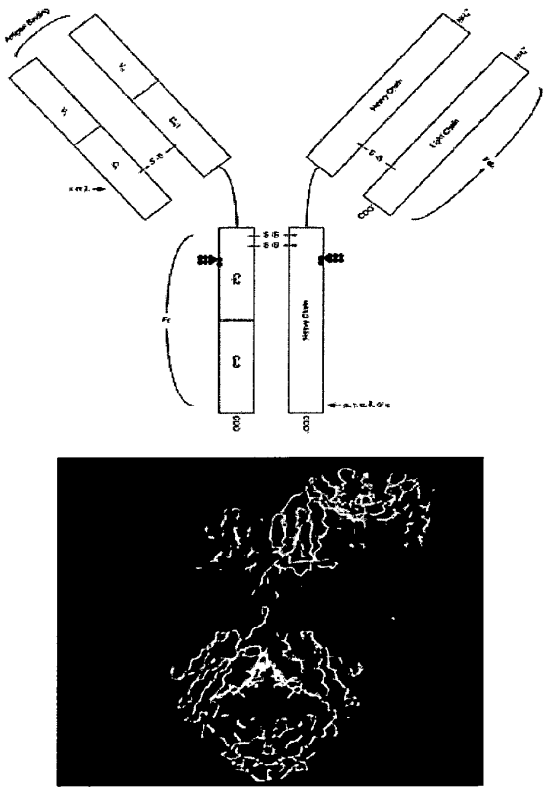


Figure 1. Linear (upper panel) and steric (lower panel) structures of immunoglobulins (IgG).

into several subclasses—IgG1, IgG2, IgG3, and IgG4 (in order of relative abundance in human plasma), with different heavy chains, named γ1, γ2, γ3, and γ4, respectively. The structural differences among these subtypes are the number and location of interchain disulfide bonds and the length of the hinge region. The light chains consist of two types—lambda (λ) and kappa (κ). In mice, the average of κ to λ ratio is 20:1, whereas it is 2:1 in humans.[6] The variable (V) regions of both chains cover approximately the first 110 amino acids, forming the antigen-binding (Fab) regions, whereas the remaining sequences are constant (C) regions, forming Fc (fragment crystallizable) regions for effector recognition and binding.[6] The N-terminal sequences of both the heavy and light chains vary greatly between different antibodies. It was suggested that the conserved sequences in human IgG1 antibodies

are approximately 95% and the remaining 5% is variable and creates their antigen-binding specificity.[5]

The V regions are further divided into three hypervariable sequences (HV1, HV2, and HV3) on both H and L chains. In the light chains, these are roughly from residues 28 to 35, from 49 to 59, and from 92 to 103, respectively.[6] Other regions are the framework regions (FR1, FR2, FR3, and FR4). The HV regions are also called the complementarity determining regions (CDR1, CDR2, and CDR3). While the framework regions form the β-sheets, the HV sequences form three loops at the outer edge of the β barrel (also see Section 2.2).

Disulfide Bonds

Most IgGs have four interchain disulfide bonds—two connecting the two H chains at the hinge region and the other two connecting the two L chains to the H chains.[6] Exceptions do exist. Two disulfide bonds were found in IgG1 and IgG4 linking the two heavy chain in the hinge region but four in IgG2.[7] In IgG1 MAb, HC is linked to the LC between the fifth Cys (C217) of HC and C213 on the LC. In IgG2 and IgG4 MAbs, it is the third Cys of HC (C123) linking to the LC.[7] A disulfide bond between HC C128 and LC C214 was found for mouse catalytic monoclonal antibodies (IgG2a).[8]

IgGs have four intrachain disulfide bonds, residing in each domain of the H and L chains, stabilizing these domains. The intrachain disulfide bonds in V_H and V_L are required in functional antigen binding.[9] Native IgG MAbs should not have any free sulfhydryl groups.[7] However, detailed examination of the free sulfhydryl groups in recombinant MAbs (one IgG1, two IgG2, and one IgG4) suggests presence of a small portion of free sulfhydryl group (approximately 0.02 mol per mole of IgG2 or IgG4 MAb and 0.03 for IgG1.[7] In rare cases, a free cysteine is found. A nondisulfide-bonded Cys at residue 105 was found on the heavy chain of a mouse monoclonal antibody, OKT3 (IgG2a).[10]

Oligosaccharides

There is one oligosaccharide chain in IgGs.[6] This N-linked biantennary sugar chain resides mostly on the conserved Asn 297, which is buried between the C_H2 domains.[5,11] For example, the oligosaccharide resides on Asn-297 of the C_H2 domain of chimeric IgG1 and IgG3 molecules[12] but on Asn 299 in a monoclonal antibody, OKT3 (IgG2a).[10] The oligosaccharide, often microheterogeneous, is typically fucosylated in antibodies produced in CHO or myeloma cell lines[5] and may differ in other cell lines.[2,11] There are many factors that dictate the nature of the glycan microheterogenity on IgGs. These include cell line, the bioreactor conditions and the nature of the downstream processing. An additional oligosaccharide can be found in rare cases. A human IgG produced by a human-human-mouse heterohybridoma contains an additional oligosaccharide on Asn 75 in the variable region of its heavy chain.[13] In addition, O-linked carbohydrates could also exist in this antibody.

Proper glycosylation is critical for correct functioning of antibodies.[11] It was demonstrated that removal of the oligosaccharide in IgGs (IgG1 and IgG3) made them ineffective in binding to C1q, in binding to the human FcγRI and activating C; and generally more sensitive to most proteases than their corresponding wild-type IgGs (one exception).[12] This is because the binding site on IgG for C1q, the first component of the complement cascade, is localized in the C_H2 domains.[11] Furthermore, the glycosylation can affect the antibody conformation.[12]

Oligosaccharides in other regions can also play a critical role. Removal of an oligosaccharide in a Fv region of the CBGA1 antibody resulted in a decreased antigen-binding activity in several ELISA systems.[13] In addition, this oligosaccharide might play critical role in reducing the antigenicity of the protein.[14]

The sugar composition of the oligosaccharide is also critical in antibody functions. It has been shown that a low fucose (Fuc) content in the complex-type oligosaccharide in a humanized chimeric IgG1 is responsible for a 50-fold higher antibody-dependent cellular cytotoxicity (ADCC) compared with a high Fuc counterpart.[15]

Heterogeneity

Purified antibodies are heterogeneous in structure. This is true for all monoclonal antibodies (MAbs) due to differences in glycosylation patterns, instability during production, and terminal processing.[5] For example, five charged isoforms were found in recombinant humanized monoclonal antibody HER2 as found by capillary isoelectric focusing (cIEF) and sodium dodecyl sulfate–capillary gel electrophoresis (SDS–CGE).[16] Six separate bands were focused under

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IEF for two mouse monoclonal antibodies IgG2a (κ) and IgG1 (κ).[17] A mature monoclonal antibody, OKT3 (IgG2a), contain cyclized N-terminus (pyroglutamic acid, −17 D) in both H and L chains, processed C-terminus (no Lys, −128 D) of the H chains, and a small amount of deamidated form.[10] Similar observation was also reported for a humanized IgG1 (κ).[18] In rare cases, gene cross-over may lead to formation of abnormal heavy chains. For example, a purified monoclonal anti-IgE antibody contains a small amount of a variant H chain, which had 16 fewer amino acid residues than the normal H chain (position is between Arg108 of the L chain and Ala124 of the H chain).[19]

Secondary and Higher-Order Structure

The basic secondary and higher-order structural features of IgGs have been reviewed.[6] Only a small portion of the three-dimensional structures of IgGs has been solved.[20] The antibody's secondary structure is formed as the polypeptide chains form anti-parallel β-sheets. The major type of secondary structure in IgGs is these β-sheets and its content is roughly 70% as measured by FTIR.[21] The light chain consists of two and the heavy chain contains four domains, each about 110 amino acid long.[6,20] All these domains have similar folded structures—β barrel, also called immunoglobulin fold, which is stabilized by a disulfide bond and hydrophobic interaction (primary). These individual domains (~12 kDa in size) interact with one another (V_H and V_L; C_H1 and C_L; and between two C_H3 domains except the carbohydrate-containing C_H2 domain) and fold into three equal-sized spherical shape linked by a flexible hinge region. These three spheres form a Y shape (mostly) and/or a T shape.[22]

The less globular shape of IgGs is maintained both by disulfide bonds and by strong noncovalent interactions between the two heavy chains and between each of the heavy-chain/light-chain pairs.[23] Through noncovalent interactions, a less stable domain becomes more stable, and thus, the whole molecule can be stabilized.[24] A detailed study indicates that the interaction between two CH3 domains are dominated by six contact residues, five of these residues (T366, L368, F405, Y407, and K409) forming a patch at the center of the interface.[25] These noncovalent interactions are spatially oriented such that variable domain exchange (switching V_H and V_L; inside-out IgG; ioIgG) induces noncovalent multimerization.[26]

The six hypervariable regions in CDR (L1, L2, L3, H1, H2, and H3) form loops of a few predictable main-chain conformations (or canonical forms), except H3 loop, which has too many variations in conformation to be predicted accurately.[27,28] There is a slight difference in the loop composition and shape between the two types of light chains.[20] However, no functional difference was found in antibodies having λ or κ chain.[6]

Basic Functions of Antibodies

The basic functions of antibodies have been reviewed.[6] There are two functional areas in IgGs—the V and C regions. The V regions of the two heavy and light chains offer two identical antigen-binding sites. The binding of the two sites (bivalent) can be independent of each other and does not seem to depend on the C region.[29] The exact antigen-binding sites are the CDR regions with participation of the frame work regions.[30] Binding of antigens seems through the induced-fit mechanism.[31,32] The induced-fit mechanism allows multispecificity and polyreactivity. It has been suggested that about 5–10 residues usually contribute significantly to the binding energy.[32]

The C regions of antibodies have three main effector functions (1) being recognized by receptors on immune effector cells, initiating antibody-dependent cell cytotoxicities (ADCC), (2) binding to complement, helping to recruit activated phagocytes, and (3) being transported to a variety of places, such as tears and milk.[6] In addition, C domains also modulate *in vivo* stability.[23,29,33] The function of Fc is affected by the structure of Fab. Variable domain exchange (switching V_H and V_L; inside-out IgG; ioIgG) affected Fc-associated functions such as serum half-life and binding to protein G and FcγRI.[26]

The hinge region provides flexibility in bivalent antigen binding and activation of Fc effector functions.[26] Two chimeric IgG3 antibodies lacking a genetic hinge but with Cys residues in CH2 regions was found to be deficient in their intermolecular assembly, and both IgG3 ΔH + Cys and IgG3 ΔH + 2Cys lost greatly their ability to bind FcγRI and failed to bind C1q and activate the complement cascade.[34]

Alternative Forms of Antibodies

In addition to species-specific antibodies, other antibody forms are generated to meet various needs. In the early development of antibody therapies, antibodies were made from murine

sources. However, these antibodies easily elicit formation of human anti-mouse antibody (HAMA). Therefore, humanized chimeric antibodies were generated. Chimeric monoclonal antibodies (60–70% human) are made of mouse variable regions and human constant regions.[2] Such antibodies can still induce formation of human anti-chimeric antibody (HACA). Highly humanized antibodies, CDR-grafted antibodies, are made by replacing only the human CDR with mouse CDR regions (90–95% human).[2] These antibodies are almost the same in immunogenicity potential as completely human antibodies, which may illicit formation of human anti-human antibody (HAHA).

Other alternative forms of antibodies have also been generated and these different forms have been reviewed.[35] Treatment with papain would cleave the N-terminal side of the disulfide bonds and generate two identical Fab fragments and one Fc fragment. Fab's are 50 kDa ($V_H + C_H1$)/($V_L + C_L$) heterodimers linked by a single disulfide bond. Treatment with pepsin cleaves the C-terminal side of the disulfide bonds and produces a F(ab)'$_2$ fragment. The remaining H chains were cut into several small fragments.[6] Cleavage by papain occurs at the C-terminal side of His-H228[36] or His-H227.[37] Reduction of F(ab')$_2$ will produce two Fab'.[23]

Fv fragments are noncovalent heterodimers of V_H and V_L. Stabilization of the fragment by a hydrophilic flexible peptide linker generates single-chain Fv (scFvs).[2] Fragments without constant domains can also be made into domain antibodies (dAbs). These scFvs are 25–30 kDa variable domain ($V_H + V_L$) dimers joined by polypeptide linkers of at least 12 residues. Shorter linkers (5–10 residues) do not allow pairing of the variable domains but allow association with another scFv form a bivalent dimer (diabody) (about 60 kDa, or trimer: triabody about 90 kDa).[38] Two diabodies can be further linked together to generate bispecific tandem diabody (tandab).[39] Disulfide-free scFv molecules are relatively stable and useful for intracellular applications of antibodies—"intrabodies."[38] The smallest of the antibody fragments is the minimal recognition unit (MRU) that can be derived from the peptide sequences of a single CDR.[2]

ANTIBODY INSTABILITY

Antibodies, like other proteins, are prone to a variety of physical and chemical degradation pathways, although antibodies, on the average, seem to be more stable than other proteins. Antibody instabilities can be observed in liquid, frozen, and lyophilized states. The glycosylation state of an antibody can significantly affect its degradation rate.[40] In many cases, multiple degradation pathways can occur at the same time and the degradation mechanism may change depending on the stress conditions.[41] These degradation pathways are divided into two major categories—physical and chemical instabilities. This section will explore the possible degradation pathways of antibodies and their influencing factors.

Physical Instability

Antibodies can show physical instability via two major pathways—denaturation and aggregation.

Denaturation

Antibodies can denature under a variety of conditions. These conditions include temperature change, shear, and various processing steps. Compared with other proteins, antibodies seem to be more resistant to thermal stress. They may not melt completely until temperature is raised above 70°C,[21,42,43] while most other mesophilic proteins seem to melt below 70°C.[44] Shear may cause antibody denaturation. For example, the antigen-binding activity of a recombinant scFv antibody fragment was reduced with a first-order rate constant of 0.83/h in a buffer solution at a shear of approximately 20,000/s.[45]

Lyophilization can denature a protein to various extents. An anti-idiotypic antibody (MMA 383) in a formulation containing mannitol, saccharose, NaCl, and phosphate was found to loose its *in vivo* immunogenic properties (only 10–20% of normal response rate) upon lyophilization.[46] Since the protein showed no evidence of degradation after lyophilization, no change in secondary structure by CD (29% β-sheet, 14% α-helix, and 57% "other"), the loss of activity was attributed to the conformational change. Indeed, tryptophan fluorescence properties were different between the lyophilized and unlyophilized antibodies.[46]

Aggregation

Antibody aggregation is a more common manifestation of physical instability. The concentration-dependent antibody aggregation was considered the greatest challenge to developing protein formulations at higher concentrations.[47] This is

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because protein aggregates generally have reduced activity and more importantly, greater immunogenicity potential because of the multiplicity of epitopes and/or conformational changes.[14,48] Immunoglobulin aggregates have been shown to cause serious renal failure[49] and anaphylactoid reactions such as headache, fever, and chills.[50] Therefore, the aggregate level in commercial intravenous immunoglobulin products is limited to less than 5% based on the WHO standards.

Aggregates can form easily both in liquid and solid states under a variety of conditions (Tab. 2). Since protein aggregation is often the consequence of protein–protein interactions, a process influenced by diffusion rate and geometric constraints of the interaction sites, such factors would influence significantly the aggregation rate, including protein concentration change, viscosity, ionic strength, pH, and temperature.[51] Other conditions may also affect protein aggregation, including shaking, long-term storage, freeze-thaw process, lyophilization process, etc.

Increasing the concentration of antibodies often increases the aggregation tendency of the protein. It was demonstrated that increasing the IgG1 concentration (in 10 mM citric acid, 100 mM NaCl pH 5.5) from 2.7 mg/mL to 50 mg/mL almost linearly increased the Nephelometric Unit (NU) from 2 to 40.[52] Since EP defines a clear solution as having equal or less than three NU, most antibody solutions in the study are opalescent except those at 5 mg/mL or less. However, as the weight average molecular weight was found to be 0.9–1.3 times that expected of a monomer (about 149 kDa), minimal protein–protein association (and readily reversible) is suggested.[52] Shaking can accelerate antibody precipitation and the shape of the precipitates may depend on the sample volume in a container.[53] It appears that protein solutions exhibiting lower surface tension are more susceptible to protein denaturation and precipitation.[53]

Low-temperature treatment may induce aggregation of antibodies. The reversible low-temperature-induced aggregation (below 37°C) of serum cryoglobulins is well known.[54] Human IgM cryoglobulin preparations easily precipitate or gel at temperatures below 10–12°C and the process is reversible at a higher temperature.[20] In a recent study, aggregation of IgG1 at low temperature at above 18 mg/mL was reversible as measured by light scattering.[52] The low-temperature-induced aggregation of cryoglobulins is poorly understood and was thought to involve sites within both Fab and Fc.[20] The authors of this article believe that low temperature reduces the hydrophobic interaction, which is the major force in protein folding. Without enough hydrophobic interaction at low temperature, hydrophobic regions of antibodies become more exposed to solvent and lead to increased intermolecular hydrophobic interaction, leading to aggregation.

In close relation to the low-temperature effect, freeze-thaw process often induces protein aggregation. However, freeze-thaw-induced antibody aggregation does not seem to be a major issue, partly due to the reversibility of antibody aggregates. Freeze-thawing of a chimeric (L6) antibody solution at pH 7.2 for as many as 35 times led to formation of dimers (not larger aggregates).[41] Maximum amount of dimers (about 20%) was observed at pH 6.5 and minimum was below 5.5 or above 8.5 (less than 2%). The freeze-thaw-induced aggregation is apparently reversible after treatment at 37°C for a few hours.[41] Freeze-thawing of rhuMAb anti-CD20 three times (freezing to either −20 or −70°C, thawing to 5°C) did not lead to significant change in aggregation, no significant loss of monomer by RP-HPLC or SEC-HPLC.[55] On the other hand, isolated Fabs seems to be more prone to freeze-thaw-induced aggregation relative to full-length antibodies. For example, Lee[56] found that freeze-thawing of a scF_v (MW 27,000 d) at 1.45 mg/mL in sodium phosphate buffered saline (PBS) at pH 7.3 dropped the %monomer content (due to aggregation) from 95% to 84%, 59%, 44%, and 37%, respectively after 1–4 cycles.

Lyophilization may induce antibody aggregation to a variable degree. It was demonstrated that lyophilization of a recombinant humanized monoclonal antibody (rhuMAb HER2), in the absence of sugars, led to a slight increase in aggregate (less than 1.2%).[57] Lyophilization caused detectable aggregation of a human monoclonal antibody in PBS or physiological saline as measured by OD600,[58] and aggregation of a mouse monoclonal antibody (MN12) [IgG2a (κ)] in the absence or presence of 5% sucrose, dextran, or hydroxypropyl-beta-cyclodextrin (HP-β-CD), as measured by visual examination.[59] In a different study, lyophilization of a monoclonal antibody-vinca conjugate in PBS at pH 7.4 caused 6.2% aggregation.[60] The lyophilization-induced aggregates could be non-covalent-linked monomers and/or even disulfide-linked.[61,62]

Antibody aggregation can easily occur during storage in a liquid state. Storing of a fully human anti-IL8 monoclonal antibody (ABX-IL8; IgG2)

Table 2. Instabilities of Antibodies

Instability	Antibody	Formulation	Condition	Effect	References
Denaturation	scFv	3 μg/mL in 10 mM phosphate-buffered saline containing 0.02% sodium azide	Shaking at a mean velocity gradient approximately 20,000/s	Loss of activity at a first-order rate of 0.83/h	45
Aggregation	Chimeric mouse/ human IgG1	2 mg/mL in PBS at pH 7.2	Stirring for 48 h with Teflon bar at 600 RPM	Increased turbidity Formation of >15 k particles (>10 μ)	91
	Human Anti-IL-8 (IgG2)	55 mg/mL, 5 mM His, pH 6	Freeze/thaw three times (room/−70°C)	About 0.6% aggregates (by SEC-HPLC)	21
	Anti-IL-8 (IgG2)	100 mg/mL, 200 mM Sucrose, 15 mM Arg, 15 mM His, pH 6	Incubation at 40°C for 6 months	About 11% aggregates (by SEC-HPLC)	21
	rhuMAbE25	10 mg/mL in excipient-free solution	Spray drying Tin = 105°C, Tout = 50–55°C	5–6% aggregate (by SEC-HPLC)	65
	MAb (type not specified)	MAb at 40 mg/mL, 85 mM sucrose, 5 mM His at pH 6, 0.01% Tween 20	Lyophilization	Cloudy (OD350 > 0.2)	1
	Human Anti-IL-8 (IgG2)	50 mg/mL, 20 mM Gly, 4 mM His, 0.2% mannitol, 16 mM Glu, 0.03% Tween 20 pH 5.7	Incubation of lyophilized form at 37°C for 1 month	4.8% aggregates (by SEC-HPLC)	21
	RhuMAb HER2	50 mg/mL in 100 mM trehalose, 5 mM His at pH 6.0	Incubation of lyophilized form at 40°C for 23 months	9.7% aggregates (by SEC-HPLC)	57
Deamidation	OKT3 (IgG2a)	1 mg/mL, PBS at pH 7.0 + Tween80	Incubation at 37°C for 2 months	90% deamidation of Asn386	10
	LA298 (IgG1)	0.5 mg/mL in 10 mM citrate at pH 7	Incubation at 25°C for 6 weeks	~12% increase in deamidation at Asn55	78
Isomerization	MAb	Lyophilized at 40 mg/mL, 85 mM sucrose, 5 mM His, 0.01% Tween 20, pH 6.0, containing 8.4% moisture	Incubation at 5°C	First-order rate constant $\sim 0.33 \times 10^{-4}$/day	1
Oxidation	rhuMAb HER2	5 mg/mL, 147 mM NaCl, 0.01% polysorbate 20, 5 mM sodium acetate at pH 5.0	5, 30, and 40°C for 2 weeks	10, 17, and 52% (total peak area)	81

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in aqueous solutions at 2–8°C or 25°C led to formation of high-molecular-weight (HMW) species.[21] Incubation of two mouse monoclonal antibodies IgG2a (κ) and IgG1 (κ) at 37°C for 32 days caused precipitation in pH 3 and 4 solutions for both antibodies.[17] Aggregation can also occur to an antibody conjugate. Storing a monoclonal antibody-vinca alkaloid conjugate (murine IgG2a) in phosphate-buffered saline solutions at 5°C between pH 4.5 and 7.4 for 1.5 months caused precipitation regardless of solution pH and aggregation was accelerated at 30°C throughout the pH range.[63]

Antibody aggregation is a common phenomenon during storage in a lyophilized state. Aggregation (OD350 > 0.2) was observed of a high concentration, lyophilized, monoclonal antibody (type not specified) by SEC under accelerated conditions.[1] So did a lyophilized chimeric monoclonal antibody during storage at 5°C (0.4% after 2 months).[64] In the absence of sugars, the extent of rhuMAb HER2 aggregation increased from about 1% to >10% after 3 months at 40°C and the rate of aggregation for these formulations was about 0.2% per month at 40°C.[57]

Antibody aggregation can readily occur in other solid forms during storage.[5] It was found that the primary degradation upon storage of a spray-dried anti-IgE (protein:mannitol = 80:20) is aggregation, and the amount of aggregates increased with increasing temperature and relative humidity (Maa et al., 1998a).[65] As much as 52% aggregate was observed by SEC-HPLC of a spray-dried anti-IgE (protein:mannitol = 80:20) after 1 year at 40°C and 38% RH (Maa et al., 1998b).[66]

Antibody aggregation is usually accelerated upon thermal treatment. In the study of chimeric (human/mouse) monoclonal antibody (BR96), Alexander and Hughes demonstrated that thermal treatment at 60°C for 166 h generated noncovalently linked aggregates.[67] In the evaluation of the relative stability of murine IgG2a (κ), chimeric IgG1(κ), trioma IgG1 (λ), and murine IgG1 (κ), Hartmann et al. demonstrated that IgG2a (κ) aggregated and precipitated after heating at 55°C for 8 h and the half-life for the loss of monomer for those four IgGs is 1, 18.1, 3.4, and 8.2 h with different aggregate species (dimer, trimer. . .) by SEC-HPLC.[68]

Surface Adsorption

Antibodies, like other proteins, can easily adsorb to a variety of surfaces. Surface adsorption can significantly reduce the antibody concentration in a solution. For example, a mouse monoclonal IgG1 has been shown to be adsorbed on the surface of glass shake flasks, causing a loss of protein in plant culture media.[69] Such a loss was minimized by coating the glass vessels or by addition of Pluronic F127.

Chemical Instability

A variety of chemical degradation pathways have been observed. These chemical degradants may or may not loose protein activity depending on the site of changes. For examples, deamidation of a monoclonal antibody, OKT3 (IgG2a) as measured by change in IEF pattern does not correlate with loss of antigen binding potency (90% deamidation of Asn386 vs. 80% activity in the potency assay).[10] In comparison, the activity of light chain deamidated recombinant humanized monoclonal antibody HER2 (rhuMAb HER2, Herceptin, trastuzuMAb) at Asn30 (to aspartate) is only 70% of the main species and that of the isomerized (heavy chain) asp102 species is only 9–21% of major species.[37] The major chemical degradation pathways in an antibody include cross-linking, deamidation, isomerization, oxidation, and fragmentation.

Disulfide Formation/Exchange

Disulfide bond formation/exchange is probably the most common cross-linking pathway, leading to chemical aggregation. This degradation pathway can occur readily in various processing steps. For example, lyophilization of a murine monoclonal antibody (IgG2a) without additives reduced the average number of –SH groups per molecule from 6.65 to 6.28, suggesting disulfide formation.[70] Since the thiolate ions are often the initiating species for this chemical degradation, increasing formulation pH usually leads to increased formation of disulfide formation. This is the case for an antifebrin T2G1s Fab′ (IgG1 (κ)), containing free –SH groups, where the percentage of dimers increased with increasing the pH from 5.8 to 9.5.[61]

Storage often leads to disulfide-based aggregation. Storage of a lyophilized excipient-free antibody (IgG1, containing 12 intra and 4 interchain disulfides) formulation for 1 year at 30°C resulted in formation of reducible dimer and trimers.[62] Similarly, reducible aggregates of a monoclonal antibody (type not specified) were detectable

by SDS–PAGE after storage of the lyophilized formulation at 50°C for 6 months.[1] Such disulfide-linked covalent dimers and trimers were also observed during storage of spray-dried anti-IgE humanized monoclonal antibody.[71]

Nonreducible Cross-Linking

Nonreducible cross-linking was observed in several studies. Incubation of a human monoclonal antibody (C23) in solution at a basic pH at 37°C for 14 days resulted in formation of nonreducible bands above the light chains at pH 4 and 10.[72] Incubation of two mouse monoclonal antibodies—IgG2a (κ) and IgG1 (κ), between pH 3.0 and 10 at 37°C for 32 days led to formation of a nonreducible 88 kDa species by SDS–PAGE.[17] A species with the same molecular weight was observed after incubation of another monoclonal antibody, OKT3 (IgG2a), at 37°C for 2 months.[10,73] Further examination suggests that this species is a cross-linked product of H and L chains between the L46-52 and H99-121 regions. Although the exact chemical nature was not determined, an oxidative step is apparently involved as inhibition of oxidation delayed the formation of this species.[10,73]

A recent study shows that a nonreducible thioether bond can be formed between C223 of the heavy chain and the C-terminal Cys residue of the light chain in humanized IgG1 antibodies. This cross-linked product has a molecular weight of 75 kDa by MS but shows an apparent molecular weight of 92 kDa by SDS–PAGE.[74]

Nonreducible cross-linked products were also observed in solid dosage forms. Incubation of a lyophilized mouse monoclonal antibody [IgG2a (κ)] without lyoprotectant or in the presence of 5% sucrose, dextran, or hydroxypropyl-beta-cyclodextrin (HP-β-CD) at 56°C for 18 days led to formation of 100-kDa band under reduced conditions by SDS–PAGE.[59]

Deamidation

Antibody deamidation has been extensively reported in the literature. This common protein degradation pathway is so prevalent that purified antibody preparations may contain many deamidated forms. For example, several deamidated forms of recombinant humanized monoclonal antibody HER2 (rhuMAb HER2, Herceptin, trastuzuMAb) were found by cation-exchange chromatography, including (1) deamidated Asn30 (to aspartate) on one light chain, (2) deamidated Asn30 (to aspartate) on two light chains, and (3) deamidated Asn55 on the heavy chain (to isoaspartate).[37] The sole conversion of Asn30 to aspartate (not isoaspartate) suggests that the deamidation mechanism in cell culture may be different from that during storage.

Storage can easily generate large amounts of deamidated products. Incubation of a monoclonal antibody, OKT3 (IgG2a) in a PBS solution at pH 7 containing Tween 80 at 37°C for 2 months led to more than 90% deamidation at Asn H386.[10] By monitoring the ammonia generation, Poborji et al.[41] estimated that 4% of the total number of Asn and Gln residues in a chimeric antibody (L6) was deamidated after storing the solution at pH 7.2 at 50°C for 3 weeks.

Deamidation also occurs in solid state. In the evaluation of storage stability of a model recombinant humanized monoclonal antibody (rhuMAb HER2), Cleland et al. reported that incubation of a high-moisture formulation (8.4% moisture) for 12 months at 40°C resulted in a 19% drop in the main peak by IEX due to deamidation.[57]

Deamidation in proteins goes mostly through the intermediate of succinimide at Asn (more readily) and Gln (see reviews[75,76]).This seems to be the case for antibodies, even though alternative mechanisms were reported, such as deamidation of Asn30 on the light chain of recombinant humanized monoclonal antibody HER2 (rhuMAb HER2, Herceptin, trastuzuMAb).[37] A method has been developed to detect the formation of succinimide.[77]

The rate of deamidation via succinimidation in proteins can be affected by a variety of factors, such as pH, sequence, and steric effect (see review[44]). A recent report demonstrated a strong pH-dependency of deamidation at Asn55 (pH 7 > pH 6 > pH 5 > pH 4) in a fully human IgG1 in the pH range of 4–7 during storage at 25°C.[78] Using small peptide as a model, Tyler-Cross and Schirch[79] found that deamidation of Asn residues depends on the amino acid residue on the carboxyl side of Asn in neutral and alkaline solutions. Increasing the size and branching of these amino acids decreased the rate of deamidation by as much as 70-fold compared with the N–G sequence (greatest rate of deamidation) but under acidic conditions, these amino acids lost their protective effect.

Isomerization

The most common isomerization in antibodies is iso-aspartic acid formation, which results not only

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from direct isomerization of Asp but also, mostly, from hydrolysis of succinimide intermediate. The pH-dependent formation of succinimide intermediate can occur either from Asn deamidation or Asp dehydration.[36] In neutral and alkaline condition, the isoAsp and Asp products formed in a peptide were at a ratio of about 3:1.[79]

Isomerization is also prevalent and difficult to control. Again, many purified proteins contain significant amount of isomerized products, either from deamidation or direct isomerization. A typical example is recombinant humanized monoclonal antibody HER2 (rhuMAb HER2, Herceptin, trastuzuMAb).[37] In addition, since isomerization occurred at Asp H102 in CDR regions, the activity of the isomerized species is only 9–21% of the major species.[37] Similarly, isomerization of Asp L32 on one light chain in a recombinant monoclonal anti-IgE antibody (E25) reduced the relative binding activity of $F(ab)'_2$ to 42% and the activity of isomerized form on both chains (iso-Asp-iso-Asp) to 15%.[36]

Since formation of the succinimide intermediate does not need participation of water, it can readily occur in solid state. Asp isomerization is one of the major degradation pathways for a lyophilized, monoclonal antibody (type not specified), and increases with increasing storage temperatures both above and below their Tg values.[1]

Like deamidation, the relative rate of isomerization can be influenced strongly by steric effect. For example, a recombinant monoclonal anti-IgE antibody (E25) contains two Asp-Gly sequences within its CDRs, but only one site was found to be labile to isomerization (Asp L32). The unreactivity of one Asp residue is suggested to be due to hydrogen bonding.[36]

Oxidation

The oxidizable residues in proteins include Met, Tyr, Trp, His, and Cys. Although oxidation is not as prevalent as deamidation and isomerization in antibodies, it can easily occur during storage of antibodies. For example, the major route of degradation of a monoclonal antibody, OKT3 (IgG2a), in solution during storage at 5°C is oxidation of a nondisulfide Cys and several Met residues.[10] Oxidation was also observed in rhuMAb anti-CD20 in solution during storage at 40°C for 2 months.[55]

Oxidation of CDP870, a pegylated one-half antibody (one light and one heavy chain) at a Met residue (M3) was also observed after incubation of the protein at 25°C or 40°C for 12 weeks (14% at pH 4.1 and 4.2% at pH 5.5).[80] Light exposure can accelerate baseline oxidation. For example, light exposure (20,000 lux for 2 weeks at 27°C) of recombinant humanized monoclonal antibody HER2, rhuMAb HER2 caused 5–10% increase in oxidation in various liquid formulations at Met H255 (primary site) and Met H431 at temperatures 30 and 40°C.[81]

Formation of Acidic or Basic Species

Antibodies can form acidic or basic species, which are often monitored by IEF or ion exchange chromatography. Among all the possibilities, deamidation is the most likely degradation pathway, leading to formation of acidic species. Therefore, formation of such species is used loosely as an indication of deamidation in the absence of other confirmatory studies. Strictly speaking, formation of such species can only be considered as an indication of chemical instability (see below). A pH-dependency is almost certain for this kind of degradation. In the stabilization of CDP870, a one-half antibody (one light and one heavy chain), which is linked through Cys to a branched (20 kDa each) PEG, Johnson et al.[80] found that the protein generated more acidic species (up to 17% at pH 4.1 and 41% at pH 5.5) by IEF as the solution pH was increased from 4.1, 4.5, 5.0, to 5.5 during storage at 25°C for 12 weeks.[80]

Another possible mechanism for generating acidic species in antibodies is the Maillard reaction, where reducing sugars can react with lysine residues. Such a reation will result in loss of positive charges and formation of apparently more acidic species. This is the case for an spray-dried anti-IgE humanized monoclonal antibody with lactose during storage.[71]

Formation of basic species was also observed in antibodies. Its formation can result from several possibilities, most likely as a result of succinimide formation, removal of sialic acids, or pyroGlu formation. Such a basic species was observed after incubation of a human monoclonal antibody (C23) at 37°C for 14 days in a solution at an acidic pH by IEF.[72]

C-Terminal Clipping

Terminal cleavages can easily occur during normal production of antibodies. For example, the majority of a purified chimeric protein rCD4-IgG lacks the heavy chain C-terminal Lys.[82]

This is also the case for recombinant humanized monoclonal antibody HER2 (rhuMAb HER2, Herceptin, trastuzuMAb)[37] and a recombinant monoclonal anti-IgE antibody (E25).[36] It was suggested that the cleavage was due to the action of basic carboxypeptidases during production.[37]

Fragmentation

The most likely sequences leading to fragmentation in proteins are Asp-Gly and Asp-Pro,[83] although other sequences can also experience cleavages, such as Asn-Ser.[79] Fragmentation can easily occur in antibodies even during production processes. A small amount of fragment (MW < LC) was detectable under reduced condition in a purified recombinant MAb IgG4 produced in Chinese hamster ovary (CHO) cells.[7] Half molecules should be monitored routinely for IgG4s.[84]

A variety of processing conditions may accelerate fragmentation of antibodies, such as acidic or basic treatment,[72] thermal treatment,[41,67] freeze-thaw,[41] and storage.[17,73,78] Fragments can simply include masses with a loss of a light chain (M-LC), a loss of a Fab arm (M-Fab), and separated heavy-chain (HC) and light-chain (LC), as well as species less than common antibody subunits, resulting from both peptide and/or disulfide bond cleavage.[67] Antibodies are also subject to radiation-induced cleavages.[85]

Maillard Reaction

Proteins may form adducts with sugars. In an investigative study, mouse MAbs were found to form glucose adducts after incubation with 0.5 M glucose at pH 7.4 for 14–21 days at 37°C and the glycated antibodies significantly increased the dissociation of the antibody-antigen binding complex.[86]

ANTIBODY FORMULATION

As mentioned above, antibodies, like other proteins, may generate a variety of degradants during production, processing, and storage both in liquid and solid states. Their tendency to generate such degradants depends very much on their individual sequence, pI, hydrophobicity, and carbohydrate content.[23] The antibody degradation products may have reduced activity and more importantly, increased immunogenicity.[14,87] A strong immune response (antibody formation) may lead to severe neutralization of the protein drug product and even endogenous proteins.

The major purpose of formulating antibodies as therapeutic agents is to control the rate of antibody degradation to provide an acceptable shelf life for worldwide shipping and storage. This can be done by choosing proper formulation excipients and other formulation conditions. It should be stressed that one formulation excipient stabilizing a specific antibody may not be suitable for another because of the differences in their sequence. A proper formulation composition should not only maximally preserve the activity of the antibody but also minimize the potential immune response, as excipients could alter the immune response by changing the presentation of the protein (such as the spacing of epitopes).[14] The following section will discuss critical factors in formulating both liquid and lyophilized antibody products. Advanced formulations will also be briefly described.

Liquid Formulation

Liquid dosage form is usually preferable to lyophilized products as it is easier to administer and less expensive to manufacture. Among all the commercial antibody products, about half are stable enough to be formulated in a liquid form (Tab. 1). Formulating a successful liquid product needs consideration of at least the following aspects.

Protein Concentration

Since the EC50 values of antibodies are often 10–1,000 times higher than for other proteins such as hormones and cytokines,[35] a relatively large amount of antibodies needs to be dosed to achieve any therapeutic effect. A large dose requires that an antibody product needs to be formulated/reconstituted at a high concentration to reduce dose volume, especially for subcutaneous injections; otherwise, a large volume of antibody preparation has to be infused. Since the aggregation process is usually concentration-dependent, antibodies at high concentrations have at least two potential issues: high tendency to aggregate during storage and likely high viscosity, leading to more difficulty during injection (injection time correlated with viscosity).[47] It has been suggested that the concentration-dependent protein aggregation is the greatest challenge to developing high-concentration

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protein formulations.[47] On the other hand, moderate increase in protein concentration may not have a significant effect. In fact, increasing the concentration of antifebrin T2G1s Fab' (IgG1 (κ)), from 0.05 to 5.0 mg/mL in solution resulted in a decrease in dimer formation during storage (73.2% to 22.9% after 13 weeks at 22°C).[61]

An effective way to reduce the viscosity of an antibody solution is to add sufficient amount of NaCl.[5,88] Adjustment of formulation pH could potentially reduce the viscosity of an antibody solution.[88] Although not very effective, histidine has been shown to reduce the solution viscosity of a fully human anti-IL8 monoclonal antibody (ABX-IL8; IgG2) from 20 to about 9 centistokes when its concentration was increased from 0 to 60 mM.[21] However, use of such excipients in reducing viscosity can be limited, as the excipient can adversely affect the stability of the antibody, such as MAb anti-CD20.[55]

Another potential issue in formulating high-concentration antibody products is their solubility limits, as presence of undissolved proteins are generally not acceptable for intravenous injection. Fortunately, the solubility of antibodies seems to be relatively high and solubility-limited formulation difficulty has rarely been reported to our knowledge. To increase the solubility of antibodies, excipients such as surfactants may be used. In a recent report, Golovanov[89] found that a combination of L-Arg and L-Glu at an equimolar concentration (50 mM) significantly increased the solubility of several proteins (either alone does not have a significant effect). In addition, the room-temperature storage stability (fragmentation) of proteins was significantly improved as monitored by SDS–PAGE.

One potential advantage of formulating a protein product at higher concentrations is the greater freeze-thaw stability. Often, proteins at high concentrations are more resistant to freeze-thaw-induced aggregation, as the interface-induced protein denaturation can easily reach a saturation point. However, it does not seem to be the case for antibodies. Increasing the concentration of a chimeric antibody (L6) did not inhibit freeze-thaw-induced aggregation.[41] In addition, surfactants such as Pluronic F68 and Tween 80 did not appreciably prevent antibody aggregation. In a different study, Tween 80 alone (or Pluoronic F68) at 0.1% failed to offer any protection against freeze-thaw-induced aggregation of a single-chain antigen-binding protein CC49/218 sF_v (MW 27,000 d) at 1.45 mg/rnL in sodium PBS at pH 7.3.[56] These results suggest that freeze-thaw-induced antibody aggregation may not be a surface phenomenon.

Effect of Formulation pH

Formulation development often starts with determination of a stable formulation pH. Like other proteins, the pH effect on the stability of antibodies depends on the formulation composition,[55] stress conditions,[41] and even antibody concentration.[84] Formulation pH may affect the physical stability of antibodies as it alters the number and distribution of charges on the protein surface. For example, low pH (below 3) caused a loss of tertiary structure in rhuMAb anti-CD20.[55] Low pH (pH 3 and 4) is also responsible for the precipitation of two mouse monoclonal antibodies IgG2a (κ) and IgG1 (κ) during incubation at 37°C for 32 days.[17] Susceptibility to pH stress varies among different IgG molecules.[68]

Regarding chemical stability, formulation pH may play a critical role in controlling many degradation pathways, such as disulfide bond formation/exchange, deamidation, fragmentation, isomerization, etc. Due to variable effects on different degradation pathways, the optimum pH value varies depending on both the sequence of an antibody and the analytical methods used in monitoring the stability. By IEF, Lam et al. found that rhuMAb anti-CD20 is most stable at pH 5 within the pH range of 3–6 irrespective of protein concentration in formulations containing 10 mM citrate, 8% trehalose, and 0.05% Tween 20.[55] By SEC-HPLC, Usami et al. found that pH 6 was the most stable condition during incubation of a human monoclonal antibody (C23) at 37°C for 14 days in a solution containing isotonic sodium chloride between pH 4 and 10.[72] Sometimes, the optimum pH values reside outside a certain range. In the study of the stability of a chimeric antibody (L6), minimum amount of dimmer formation was observed at pH below 5.5 or above 8.5 (less than 2%) during freeze-thaw.[41]

It needs to be mentioned that oxidation could be inhibited by adjusting formulation pH. In a formulation containing 40 mg/mL MAb anti-CD20, 150 mM trehalose, 0.9% benzyl alcohol, 0.02% Tween 20, and 50 mM histidine, reducing the pH from 7.5 to 5 virtually eliminated the oxidation during storage at 40°C for 2 months.[55] In a different study, oxidation (M3) of PEG-CDP870, a one-half antibody, in a formulation containing 160 mg/mL CDP870, 10 mM sodium acetate, and

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125 mM NaCl was slower at pH 5.5 than at pH 4.1 (4.2% vs. 14% after 12 weeks at 40°C).[80]

Due to the simultaneous presence of multiple antibody degradation pathways, the formulation pH has to be adjusted to balance all the potential degradations. Generally, a weakly acidic condition seems to be the optimum for most antibodies (see Tab. 1).

Effect of Buffering Agents

It has been long recognized that both the type and concentration of a buffering agent may affect the stability of proteins (see review[44]). For example, phosphate buffer accelerated deamidation of an IgG1 relative to citrate buffer at both pH 6.5 and 7.0 during storage at 25°C.[78] Therefore, proper choice of a buffering agent can potentially achieve both pH control and stabilization of antibodies. In rare cases, a buffering agent good for one antibody may be harmful to another. One of these agents is histidine, which has a pKa of pH 6 and potentially good in controlling a weakly acidic condition. It has been shown to provide better protection against precipitation for a fully human anti-IL8 monoclonal antibody (ABX-IL8; IgG2) upon thermal incubation both at 50 and 40°C than citrate at pH 6.[21] Addition of a small amount of histidine (0.4 mM) also increased the freeze-thaw (4×) recovery of a single-chain antigen-binding protein CC49/218 sF_v (MW 27,000 d) at 1.45 mg/rnL in PBS solution at pH 7.3 from 37% to 90%.[56] On the contrary, histidine was found not only to increase the rate of aggregation of rhuMAb anti-CD20 during storage but also to induce coloration of the solution compared to a acetate-buffered antibody solution at pH 5.[55] In addition, oxidation of Fc was also faster in histidine solution.

The concentration effect of a buffering agent was demonstrated in a study where freeze-thaw-induced aggregation of a chimeric antibody (L6) was higher with increasing phosphate buffer concentration (0.01 vs. 0.05).[41] Therefore, both the type and concentration of buffering agents need to be examined.

Effect of Formulation Excipients

Use of formulation excipients remains a major and convenient approach in stabilizing antibodies in solutions. It is so at least in reducing antibody aggregation.[47] A variety of formulation excipients have been shown to stabilize antibodies under different processing conditions and during storage, including sugars,[41] polyols,[41] amino acids,[41,84] surfactants,[84] and polymers.[45,84]

Formulation excipients are effective generally in protecting the physical stability of an antibody. Among these excipients, sugars are the most commonly used. A variety of sugars, such as sucrose, trehalose, glucose, have been shown to reduce the freeze/thaw-induced aggregation of rhuMAb anti-CD20,[55] a chimeric antibody (L6),[41] a human anti-IL8 monoclonal antibody (ABX-IL8; IgG2),[21] and a single-chain antigen-binding protein CC49/218 sF_v (27 kDa).[56] Other excipients can be equally or even more effective in protecting antibodies.. Histidine was shown to be equally effective as sucrose in reducing freeze-thaw induced aggregation of a human anti-IL8 monoclonal antibody (ABX-IL8; IgG2) and combination with Arg resulted in a better protection than sucrose in solution stability at different incubation temperatures.[21] Sugars are not always effective. In the evaluation of freeze-thaw stability of a single-chain antigen-binding protein CC49/218 sF_v (MW 27 kDa) at 1.45 mg/mL in sodium phosphate buffered saline (PBS) at pH 7.3, addition of 10 mM trehalose, lactose, glucose, or mannitol did not offer any protection.[56] In a rare case, sucrose has been shown to promote agitation-induced aggregation of an IgG1 antibody.[90]

Formulation excipients may inhibit chemical degradation of antibodies. Replacing 0.14 M NaCl with 8% trehalose or 4% mannitol in a rhuMAb anti-CD20 increased the chemical stability as measured by IEX during storage at 40°C.[55] Such an effect is likely a result of stabilization of antibody conformation. Some excipients may protect antibodies against oxidation. Replacing NaCl with 4% mannitol and 1% benzyl alcohol protected recombinant humanized monoclonal antibody HER2, rhuMAb HER2, against oxidation (primarily Met-255) at 30 or 40°C.[81] This effect is likely due to a function of mannitol as a free radical scavenger. Some commonly used antioxidants, such as thiosulfate and methionine, are effective in inhibiting antibody oxidation. The minimum effective levels (molar ratios of antibody to antioxidant) required to inhibit temperature-induced oxidation of rhuMAb HER2 were 1:5 and 1:25 for methionine and thiosulfate, respectively.[81] However, use of thiosulfate has led to formation of a thiosulfate adduct of rhuMAb HER2.[81] The safety of such a thio-containing adduct has not been reported.

One of the main excipients often used in antibody formulation is surfactants. They are usually

effective in reducing shaking/stirring-induced aggregation[91] but may have negative effect during long-term storage.[92] One obvious concern is that such compounds may contain a residual level of peroxides, which have been shown to cause protein oxidation.[93] Therefore, use of surfactants should be kept to a minimum in an antibody formulation if possible. For example, removing Tween 20 in a recombinant humanized monoclonal antibody HER2, rhuMAb HER2 solution reduced the oxidized antibody from 52% to <10% at 40°C.[81]

Effect of Shaking/Shearing

Both shaking and shearing can denature antibodies. It was found that shaking murine antibody-based products caused antibody precipitation and the shape of the precipitates are different depending on the fill volume.[53] In the evaluation of shear effect on the antigen-binding activity of a recombinant scFv antibody fragment in a buffer solution, Harrison et al.[45] found that the binding activity decayed at a mean rate constant of 0.83/h at a shear of approximately 20,000/s.

Effect of Preservatives

A limited number of studies have been conducted on the compatibility of preservatives with antibodies. Compatibility depends both on the antibody and the preservative. In the evaluation of the effect of several preservatives, including benzyl alcohol, chlorobutanol, methylparaben, propylparaben, phenol, and m-cresol, on the stability of a humanized monoclonal antibody, Gupta and Kaisheva[94] found that the antibody was most stable in the presence of methylparaben and propylparaben, compatible with benzyl alcohol and chlorobutanol at low concentrations, and not compatible with phenol and m-cresol, as measured by a variety of assays, including SEC-HPLC, DSC, light scattering, UV, and potency assay.

Effect of Processing Equipment

Another important issue in antibody formulation is the choice of proper processing equipment/containers. A few studies have shown that antibody stability can be significantly affected if an inappropriate choice is made. In the study of the stability of recombinant humanized monoclonal antibody HER2, rhuMAb HER2, in liquid formulations, Lam et al.[81] found that replacing the stainless steel filler with a nonstainless steel filler reduced the oxidized antibody (primarily Met-255) from 52% to 18% after storage for 2 weeks at 40°C. It was suggested that Fe ions, released from erosion of stainless steel by chloride ion at low pH, catalyzed the oxidation. In a similar case, Chen et al.[21] demonstrated that a fully human anti-IL8 monoclonal antibody (ABX-IL8) in aqueous formulations containing 60 mM His had a higher level of aggregates after freeze-thaw in a stainless steel vessel and subsequent storage at 40°C, possibly due to the effect of Fe ions generated from a similar mechanism. In addition, proteins have been shown to facilitate metal corrosion.[95]

Effect of Product Containers

The effect of product containers was clearly demonstrated in a recent study. McCormick et al.[96] reported that Tween 80, intended to replace human albumin in the original formulation, interacted with uncoated rubber stoppers in single-use Eprex syringes. The stoppers leached a small amount of plasticizers in the drug solution, which acted as an adjuvant, and caused a strong IgG response to the recombinant human erythropoietin. The immune response was significantly minimized later by switching to PTFE-coated rubber stoppers. In addition, headspace oxygen can accelerate oxidation of antibodies and replacement with an inert gas could minimize such a reaction.[73]

Accelerated Stability Studies

To accelerate formulation development process, accelerated stability studies are usually conducted. However, estimation of room temperature or 5°C stability based on accelerated stability studies for antibodies, like other proteins, may or may not be possible or accurate. The unpredictability is due to the presence of complex and multiple degradation pathways, which may have different degree of temperature dependencies.[10] Kroon et al.[10] found that relative to other degradation pathways of a monoclonal antibody, OKT3 (IgG2a), Asn deamidation was preferentially accelerated with increasing temperature. Therefore, caution needs to be applied in interpreting such high-temperature-induced deamidation. On the other hand, Poborji et al.[41] found that loss of monomeric chimeric antibody (L6) at pH 7.2 followed Arrhenius behavior in the temperature range of 30–50°C and extrapolation of the high temperature SEC data to 2–8°C at pH 7 and

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5.5 resulted in comparable stability estimates with the available real-time data.

Lyophilized Formulation

Preparation of lyophilized protein formulations has been extensively reviewed.[97–101] Like most proteins, some antibodies are not stable enough in a liquid form and lyophilized dosage forms will have to be considered. Lyophilization was suggested to be one of the two approaches in solving antibody aggregation.[47] This section will discuss some critical issues in formulating a lyophilized antibody product.

Amorphous Versus Crystalline State

It has been commonly believed that proteins are more stable in an amorphous state.[103] This is mainly because proteins need another excipient(s) to replace water molecules to from the required H-bonds as water molecules are removed during drying and a crystalline matrix does not provide intimate H-bonding between the excipient and the protein.[104,105] It appears that if the protein itself can be crystallized during the lyophilization process (may be difficult practically), the protein could be more stable and might not need as much stabilizing agent as in the amorphous state. In a recent report, Shenoy et al.[106] compared the stability of two model proteins, glucose oxidase (GO) and lipase, and demonstrated that dry crystalline formulations are significantly more stable than their amorphous counterparts as monitored by size-exclusion chromatography. It remains to be seen whether this can be applicable to antibodies.

Effect of Formulation Excipients

To make a lyophilized antibody product, at least one formulation excipient, a bulking agent, is needed to confer an acceptable cake. If the antibody is not stable enough in the presence of a bulking agent during lyophilization or storage, a stabilizer(s) is included. The relative amount of formulation excipients (bulking and stabilizing agents) should be carefully chosen as these agents may affect each other's behavior, the tonicity, and stability of the lyophilized products.

Commonly used bulking agents include mannitol and glycine. Crystallization of these agents during lyophilization makes them wonderful bulking agents but poor stabilizing agents.[57] Although mannitol could stabilize an antibody as in the case for a monoclonal antibody-vinca conjugate in a PBS solution at pH 7.4 upon lyophilization,[60] many studies showed that mannitol could not stabilize effectively or even destabilizes antibodies. Such examples include the aggregation of a lyophilized model recombinant humanized monoclonal antibody (rhuMAb HER2) in the presence of mannitol during storage.[3,57] In the lyophilization of a human monoclonal antibody at 1 mg/mL, it was demonstrated that addition of 5% mannitol has no effect on the lyophilization-induced aggregation in PBS or physiological saline as measured by OD600 and addition of 10% mannitol actually accelerated aggregation during the lyophilization process.[58] On the other hand, when used in combination with sucrose or trehalose, similar degree of stabilization to that for sugar alone may be achieved such as the case for model recombinant humanized monoclonal antibody (rhuMAb HER2).[57] The disaccharide/mannitol formulations also inhibited deamidation of this antibody during storage to a greater extent than the lyoprotectant formulations alone.[57]

Sugars are the most common stabilizing agents used in lyophilized formulations. Among these, two nonreducing sugars, sucrose and trehalose, are usually the first choice. Both sugars protected the stability of a few lyophilized antibodies equally well. These antibodies include lyophilized recombinant humanized monoclonal antibody, rhuMAb HER2,[57] IgG1 (containing 12 intra and 4 interchain disulfides),[62] anti-IgG,[107] and chimeric monoclonal antibody.[64] However, sucrose seemed to be more effective in protecting a lyophilized IgG1 against aggregation during storage at 40°C at a sugar:protein weight ratio of 1:1 (Chang et al. 2005b).[102] In another study, sucrose at 5% failed to offer any protection to a lyophilized mouse monoclonal antibody (MN12) (IgG2a (κ)) during storage at 56°C for 18 days.[59] Again, as mentioned in the liquid formulation section, even the most commonly used stabilizers may not stabilize all antibodies in the solid state and antibodies need to be treated as individually different molecules.

When sugars are used as stabilizing agents (lyoprotectants), a sufficient quantity needs to be used to achieve a significant effect depending on the protein concentration. It has been suggested that a molar lyoprotectant:protein ratio of 300:1 or greater should be used.[47] Indeed, the available literature data indicate a ratio higher than 300 is needed for better stabilization. It was shown that sugars protect the stability (aggregation and Asp isomerization) of a lyophilized, monoclonal

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antibody (type not specified) to a significant level when the sugar to protein molar ratio was increased to 500:1, which happens to the theoretical number of water binding sites in the molecule.[1] Similarly, Andya et al.[62] demonstrated that use of sucrose or trehalose inhibited the aggregation of a lyophilized antibody (IgG1—containing 12 intra and 4 interchain disulfides) during storage at 30°C to a maximum degree when the sucrose/trehalose:protein ratio was 500 or higher, which is roughly equivalent to the number of water-binding sites on the surface of the protein (550 estimated). Increasing the trehalose/anti-HER2 ratio from 60 mM (360 molar ratio) to 200 mM (1,200 molar ratio) significantly increased the stability (monomer by SEC-HPLC) of lyophilized formulation at 25 mg/mL at 40°C.[107] In a recent report, it was found that at least a weight ratio of 2:1 (molar ratio of about 800) was needed to achieve a maximum protection of a lyophilized sucrose:IgG1 formulation against both aggregation and chemical degradation during storage at 40°C (Chang et al. 2005a).[101]

Other formulation excipients were also used and some of these are effective in stabilizing certain antibodies. A recent study demonstrated that addition of sorbitol to a sucrose or trehalose formulation resulted in further enhancement of storage stability of a lyophilized IgG1 antibody (Chang et al. 2005b).[102] Either dextran or HB-β-CD (better) protected the activity of a lyophilized mouse monoclonal antibody (MN12) (IgG2a (κ)) during storage.[59] Glucose was shown to inhibit gamma irradiation-induced fragmentation of humanized monoclonal antibodies (hr3).[85]

Effect of Formulation "pH" and Buffering Agents

The pH of the liquid formulation can have a potential effect on the stability of the lyophilized formulation, even though the solid-state "pH" could not be well defined. In the evaluation of the stability of a lyophilized monoclonal antibody-vinca conjugate, aggregation seems to be lower at a neutral pH 7.1 than at either pH 8.5 or 6.1 when the lyophilized formulation was stored at 25°C.[60] In adjusting the formulation pH, use of a buffering agent may have effect on the antibody stability both during lyophilization and storage of the lyophilized products. For example, in the stability study of a fully human anti-IL8 monoclonal antibody (ABX-IL8; IgG2), Chen et al.[21] demonstrated that addition of histidine inhibited the formation of HMW species upon lyophilization and during storage at 37°C in a concentration-dependent manner. Among several excipients including His, Gly, mannitol, Glu, and polysorbate 20, His is the most critical in protecting solid-state stability of the antibody (minimizing aggregation). In a different study, the aggregation rate of a lyophilized anti-IgE at 5°C at 5 mg/mL in 10 mM buffer is affected both by pH and buffers in the following order: Na Phosphate, pH 7 > K Phosphate, pH 7 > Na Succinate, pH 6 or 5 > Histidine, pH 7.[107] It should be noted that some buffering agents may induce significant pH change due to selective crystallization during lyophilization, such as sodium phosphate. Such buffering agents should be avoided in preparing pH-sensitive antibody formulations.

Effect of Protein Concentration

In many cases, increasing the protein concentration increases the stability of the protein during lyophilization (see review).[98] However, antibodies do not seem to follow this trend and many antibodies have been shown to be less stable both during lyophilization and storage at high concentrations. For example, lyophilization of a human monoclonal antibody caused significant aggregation of MAb formulated in PBS or physiological saline as measured by OD600 and increasing the protein concentration from 1 to 2.5 or 5 mg/mL increased the OD600 from 0.194 to 0.311 or 0.427, respectively.[58] Similar trend was also observed during storage of lyophilized formulation. Increasing the concentration of a chimeric monoclonal antibody from 2.5 mg/mL to 25 mg/mL in a lyophilized formulation, containing 31 mg/mL sucrose, 15 mM NaCl, 0.02% Tween 80, 10 mM citrate, increased the aggregate level from 0.4% to 0.6% after 2 months at 5°C.[64] In the evaluation of the stability of a model recombinant humanized monoclonal antibody (rhuMAb HER2), Cleland et al.[57] demonstrated that increasing the protein concentration from 25 mg/mL to 50 mg/mL in a lyophilized formulation (containing 60 mM trehalose, 5 mM His at pH 6) increased the aggregate level from 8.5% to over 30% by SEC-after storage at 40°C for 44 months. Such behavior was also observed of anti-HER2 from 5 to 21 mg/mL containing 250 mannitol.[107]

Effect of Moisture Content

The effect of moisture on the stability of lyophilized antibodies has not been consistent and seem

to depend on the antibody and the degradation pathways being monitored.[57] Generally, increasing moisture content of a lyophilized antibody will gradually increase the rate of degradation during storage. Such negative effect of moisture was clearly demonstrated in a variety of degradation pathways, including dimerization of T2G1s Fab' (IgG1 (κ)) in a moisture content range of 1.5–12.3%,[61] aggregation of TNF-MAb between 0.68% and 8%,[108] formation of acidic/basic species of rhuMAb HER2 between 1% and 8.4%[57] and fragmentation of a mouse monoclonal antibody (MN12) with and without secondary drying.[59]

In other cases, the effect of moisture is either complex or not significant. For example, Breen et al.[1] found that high-moisture formulations (8%) of a lyophilized monoclonal antibody (type not specified) showed a higher rate of aggregation, fragmentation, and Asp isomerization during storage than those containing 2% or 5% moisture but formulations containing intermediate moisture levels (2–3%) were less prone to aggregation than those containing a lower moisture levels (1%) as measured by SEC under accelerated storage conditions. Similarly, the aggregation of a lyophilized IgG1 was found to be minimal at an intermediate moisture level around 3% at different storage temperatures. (Chang et al. 2005b).[102] In a different study, Cleland et al.[57] found that using a model recombinant humanized monoclonal antibody (rhuMAb HER2), a residual moisture between 1 and 8.4% had no impact on the rate of aggregation during storage at 2–8 or 40°C for 12 months.

Accelerated Stability Studies

Like antibodies in a liquid state, those in a solid state may or may not undergo degradation that is characteristic of Arrhenius behavior. It is generally believed that stability studies should be conducted at least below the glass transition temperature of the formulation. Duddu et al.[64] found that accelerated stability data generated in the glassy state (40°C) seems to be a better predictor of the relative stability of formulations than the data generated at a higher temperature (60°C). In the study of the stability of a high-concentration, lyophilized, monoclonal antibody (type not specified), Breen et al.[1] demonstrated that chemical and physical degradation pathways followed Arrhenius kinetics during storage in the glassy state. Only Asp isomerization followed the Arrhenius model above the Tg value.

Advanced Formulations

In addition to the liquid and lyophilized dosage forms on the market, other dosage forms have been tried and limited success has been reported.

Spray-Dried Formulations

Spray drying is an efficient way of preparing solid dosage forms because of the rapid removal of a solvent(s) from a drug solution. It is a very attractive alternative to lyophilization in preparing solid protein formulations. So far, a number of studies have been conducted for proteins and partly because of the high-temperature exposure in the process, albeit very short, limited success has been achieved. Protein aggregation during spray drying seems to be the major challenge.[65,66,109,110]

Spray drying of antibodies has also been tried. As for other proteins, process-induced protein aggregation seems to be the major issue.[71,110] Careful selection of proper excipients could minimize this problem. In the evaluation of a spray-dried formulation for a rhuMAbE25, it was demonstrated that trehalose or lactose minimized the aggregation during spray drying (aggregate dropped from 5–6% to ≤1%). For trehalose, the molar ratio for significant inhibition of aggregation is at or above 300:1 to 500:1 (excipient: protein).[71] Such a ratio seems to be comparable with that used in the lyophilized formation. The addition of mannitol also reduced aggregation, but only up to a molar ratio of 200:1 and further increase in mannitol content resulted in crystallization, which had a detrimental effect on protein stability and aerosol performance.[71] In a different study, either trehalose or sorbitol could inhibit IgG aggregation during spray drying.[110]

Stabilized Antibodies via Mutagenesis

Antibody fragments, such as scFv, are alternative antibody therapeutics. However, since their stability is not as good as full-length antibodies, mutagenesis, or chemical modifications have been tried to improve its stability. For example, R71A mutagenesis in the heavy chain of an unstable scFv dramatically increased its stability when incubated in human serum while having only a minor effect on the binding affinity of the molecule.[111] A more stable heavy chain, made through mutation, can increase the stability of

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the light chain, and thereby confer more stability to the whole scFv.[24]

Stabilized Antibodies via Chemical Modifications

The clinical performance of an antibody can be desirably changed by conjugation of the antibody to another entity. For example, McIntosh et al.[112] demonstrated that conjugation of carboxymethyl dextran to a tumor-antigen-specific monoclonal antibody (A5B7) increased the clearance of the antibody in mice but conjugation of cisplatin (*cis*-dichlorodiammine platinum II) with the antibody complex restricted its tissue distribution and reduced its systemic clearance.

PEGylated Antibodies

A common method of increasing plasma half-life of proteins is to increase the size of the protein by attaching a PEG moiety. Currently, there are at least four PEGylated commercial protein products on the market. However, the long plasma half-lives (as high as 7–23 days) of antibodies made much less appealing the development of such an antibody product. Since the long half-life was due to the binding of Fc to the Fc-receptor (neonatal Fc receptor, FcRn) in various tissues,[33,35] antibody fragments, such as Fab′ lacking the Fc portion have a much shorter half-life and could be good candidates for PEGylation. Such a modification was made on a humanized IgG Fab′ fragment via site-specific attachment to an additionally engineered Cys residue, as modification via the lysine residues led to substantial losses in binding activity.[29]

Controlled Release Systems

Controlled release systems have been extensively investigated for proteins in the past two decades.[113,114] However, limited work has been conducted in this area for antibodies, partly due to their relatively long plasma half-lives compared with other proteins. To achieve long-term effect, a controlled released system consisting of biodegradable poly (lactic-glycolic) acid (PLGA) was prepared for an anti-cocaine catalytic monoclonal antibody and an *in vivo* release of the antibody for up to 10 days was demonstrated in mice following subcutaneous injection.[115] It is anticipated that such systems could be useful if a minimum of 1-week duration of release is achieved.

SUMMARY

Antibodies have similar tertiary structures. The presence of a significant number of disulfide bonds and intimate domain–domain interactions in antibodies make them relatively stable and more resistant to moderate thermal stress compared to other proteins. Nevertheless, antibodies do experience a variety of instabilities similar to most proteins. These physical and chemical instabilities include denaturation, aggregation, surface adsorption, deamidation, oxidation, isomerization, fragmentation, etc. Due to the significant difference in the primary sequence among different antibodies, the relative severity of these degradation pathways can be significantly different. On the average, aggregation, deamidation, and isomerization seem to be more prevalent in antibodies.

Partly due to the stability difference, some antibody products are made into a liquid form while others are lyophilized (Tab. 1). The solution pH of these commercial products is either neutral or weakly acidic, suggesting that such a pH range is probably optimal for most antibodies. Surfactants have been used in most of these antibody products, presumably to inhibit antibody aggregation, and will likely be used in other antibody drug candidates. Two most commonly used formulation excipients in these antibody formulations are sucrose and NaCl. For liquid formulations, either one can be used if they offer the same level of stability protection. For lyophilized formulations, sucrose is preferable as NaCl does not form intimate H-bonds with the antibody and also reduce the glass transition temperature of formulation, making lyophilization less efficient. All the formulation excipients and buffering agents used in commercial antibody products or discussed in the article should be evaluated individually for each antibody drug candidate through stability studies before they are chosen as part of the product, as antibodies are structurally different.

The available stability data in the literature and the information on the commercial antibody products suggest that antibody formulation seems challenging but relatively manageable, compared to other protein drugs. Three major issues in antibody formulation are apparently challenging and need significant attention in the coming years: (1) development of stable high-concentration formulations, (2) management of the high-concentration-associated viscosity of these formulations,

and (3) minimization of antibody-formulation-induced immune response.

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ORIGINAL ARTICLE

Effect of Eculizumab on Hemolysis and Transfusion Requirements in Patients with Paroxysmal Nocturnal Hemoglobinuria

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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.

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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.

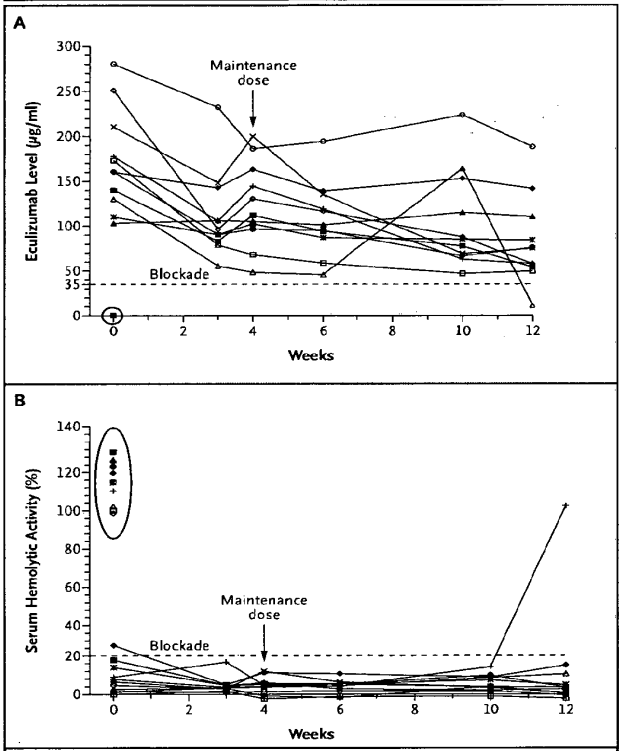


Figure 1. Pharmacokinetic and Pharmacodynamic Analysis of Eculizumab in 11 Patients with Paroxysmal Nocturnal Hemoglobinuria (PNH).

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).

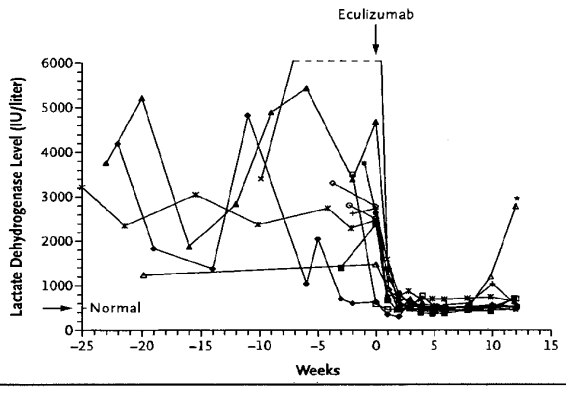


Figure 2. Analysis of Lactate Dehydrogenase Levels, a Biochemical Indicator of Hemolysis, in 11 Patients with Paroxysmal Nocturnal Hemoglobinuria up to 25 Weeks before and during 12 Weeks of Eculizumab Treatment.

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).

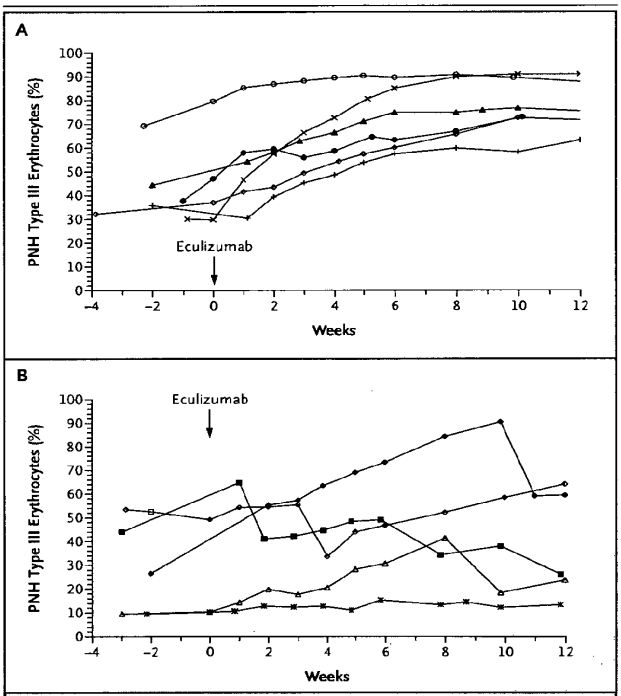


Figure 3. Changes in the Percentage of Paroxysmal Nocturnal Hemoglobinuria (PNH) Type III Erythrocytes during Eculizumab Treatment in Six Patients Who Received No Transfusions during Treatment (Panel A) and Five Patients Who Received at Least One Transfusion after Starting Treatment (Panel B).
The initial 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

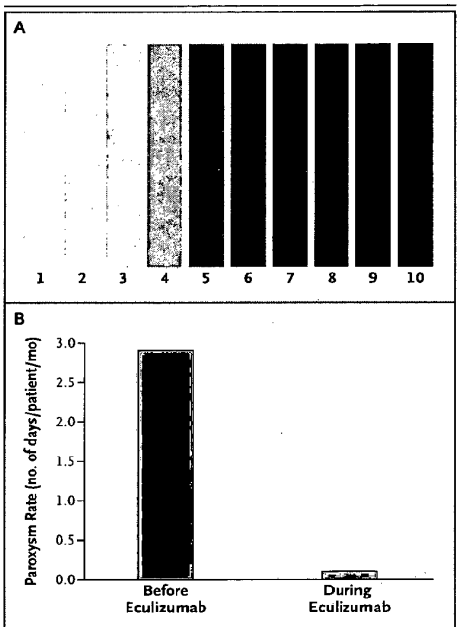


Figure 4. Incidence of Paroxysms during Eculizumab Treatment.

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.

Funded by Alexion Pharmaceuticals, Cheshire, Conn.

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.

We are indebted to Drs. Leonard Bell, Stephen Squinto, and Paul Finnegan for critical review of the manuscript; to Karen Hannon for excellent technical support; and to Jan Farish, David Buchanon, Pilar Hernandez-Campo, and the nurses of Healthcare-at-Home for their assistance in carrying out the study.

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Date
10.08.2017

Reference	Application No./Patent No.
P2913 EP/1	11001632.6 - 1466 / 2359834

Applicant/Proprietor
Alexion Pharmaceuticals, Inc.

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Date: 14.08.2017

Reference	Application No./Patent No.
AMG17203OP OPPO 01	11001632.6 - 1466 / 2359834
Applicant/Proprietor: Alexion Pharmaceuticals, Inc.	

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Subject:
- ☒ Your letter of 08.08.2017
- ☐ Our telephone conversation of
- ☐ Communication of
- ☐

Enclosure(s):
- ☐ Letter from the proprietor of the patent of
- ☐ Letter from the opponent of
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ADVANCES IN HEMATOLOGY

Current Developments in the Management of Hematologic Disorders

Section Editor: Craig M. Kessler, MD

Eculizumab for the Treatment of Paroxysmal Nocturnal Hemoglobinuria

Anita Hill, MBChB(Hons), MRCP, DipRCPath
Clinical Research Fellow
Department of Haematology
Leeds Teaching Hospitals NHS Trust

H&O Can you give a short background on the standard of care for paroxysmal nocturnal hemoglobinuria before the advent of eculizumab?

AH Until recently, the treatment for paroxysmal nocturnal hemoglobinuria (PNH) was predominantly supportive. Red blood cell (RBC) transfusions were administered when appropriate, and warfarin or other anticoagulation therapy was given following a thrombosis. In the UK we practice primary prophylaxis with anticoagulant therapy depending on the neutrophil PNH clone size. Other therapies, such as corticosteroids or androgen therapy, have also been used, but less commonly and with efficacy in only a few patients; the mechanism of action behind these treatments is unclear. Stem cell transplantation has also been used in the past, but of course this approach is associated with serious complications. It has been difficult to develop an evidence base for treatment because of the heterogeneous nature and rarity of this disease. Before eculizumab (Alexion), treatment for PNH was limited to alleviating symptoms and improving patient quality of life. Eculizumab is a monoclonal antibody that blocks the complement system at protein C5.

H&O What is the rationale for the development of eculizumab for the treatment of PNH?

AH We know the hemolysis in PNH is complement-mediated, thus this drug should be effective in preventing hemolysis. The pathogenesis of PNH is known to be due to complement sensitivity of RBCs against complement attack. RBCs in PNH have lost their protective proteins (eg, CD55 and CD59) that would normally prevent complement-mediated lysis. It was hypothesized that if the complement attack on the RBCs could be blocked, the hemolysis in PNH and its associated symptoms could be prevented. Based on patients who have inherited complement deficiencies, it appears that patients with deficiencies in complement proteins C5–C9 suffer few clinical consequences compared to those with deficiencies in the complement proteins before C5. Therefore, C5 seemed to be a logical target for blocking the complement cascade. Eculizumab was developed for the purpose of blocking the complement system safely and without severe complications.

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AH The research strategy has been to develop therapeutic approaches that block complement activity, which contributes to a variety of disorders. There is some evidence that some components of autoimmune disorders such as rheumatoid arthritis and systemic lupus erythematosus are complement-mediated. In PNH, however, almost all of the clinical symptoms are due to the uncontrolled activity of complement. Therefore, whereas there are many possible disorders in which complement blockade might have some efficacy, PNH is the disorder in which we had little doubt that eculizumab would be effective.

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Section Editor: Craig M. Kessler, MD

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August 14, 2017

European patent 2 359 834 (Application No. 11 001 632.6)
Title: Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement - EP 2 359 834
Proprietor: Alexion Pharmaceuticals, Inc.
Opponent: Amgen Inc.
Our Ref.: AMG17203OP

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The document **XP001538426**; HILL ANITA, "Eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria", *CLINICAL ADVANCES IN HEMATOLOGY & ONCOLOGY*, MILLENNIUM MEDICAL PUB., NEW YORK, NY, US, (20051101), vol. 3, no. 11, ISSN 1543-0790, pages 849 – 850, is enclosed herewith.

Respectfully,

Solveig Moré

Dr. Solveig Moré
European Patent Attorney

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Dr. rer. nat., Dipl.-Phys.

René Okoampah [1234]
Dipl.-Ing.

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Judith Hesse [8]
LL.M.

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LL.M.

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Dr. rer. nat., Dipl.-Phys.

1 European Patent Attorney
2 European Trademark Attorney
3 European Design Attorney
4 Patentanwalt
5 Solicitor (England & Wales)
6 U.S. Patent Attorney
7 U.S. Patent Agent
8 Rechtsanwalt

Partnerschaftsregister des AG München
PR-Nr. 1278

Samsung Bioepis Exhibit No. 1002
Page 6096

Europäisches Patentamt
European Patent Office
Office européen des brevets

Submission in opposition proceedings

Representative:

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- representing the opponent(s):	Amgen, Inc.
Opponent/representative's reference	AMG17203OP

The information given below is pertaining to the following patent in opposition proceedings:

Patent No.	EP2359834
Application No.	EP11001632.6
Title of the invention	Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement
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 - Submission regarding document D6	AMG17203OP_Filing_D6.pdf	OTHER-1.pdf

Evidence filed subsequently:

D6	Non-patent literature - article	Hill, "Eculizumab for the Treatment of Paroxysmal Nocturnal Hemoglobinuria" Clinical Advances in Hematology & Oncology, 2005 original file name: D6 - Hill .pdf attached as: Published-Evidence-1.pdf

Signatures

AMG17203OP

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Page 6097

Place:	**Munich**
Date:	**14 August 2017**
Signed by:	**Solveig Moré 24886**
Representative name:	**Solveig MORÉ**
Capacity:	**(Representative)**

Europäisches Patentamt
European Patent Office
Office européen des brevets

European Patent Office
80298 MUNICH
GERMANY

Questions about this communication ?
Contact Customer Services at www.epo.org/contact

Dörries, Hans Ulrich
df-mp
Fünf Höfe
Theatinerstrasse 16
80333 München
ALLEMAGNE

089 – 210296 33

Date: 14.08.2017

Reference	Application No./Patent No.
AMG17203OP OPPO 01	11001632.6 - 1466 / 2359834
Applicant/Proprietor: Alexion Pharmaceuticals, Inc.	

BRIEF COMMUNICATION

Subject:
- ☒ Your letter of 08.08.2017
- ☐ Our telephone conversation of
- ☐ Communication of
- ☐

Enclosure(s):
- ☐ Letter from the proprietor of the patent of
- ☐ Letter from the opponent of
- ☒ Copy (copies) D6

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 enclosed a copy of document D6 (XP001538426).

For the Opposition Division

Europäisches Patentamt • European Patent Office • Office européen des brevets

☐ **Registered letter**

Samsung Bioepis Exhibit No. 1002
Page 6099

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	5533370
Application number	EP11001632.6
Patent number	EP2359834
Date of receipt	14 August 2017
Your reference	AMG17203OP
Opponent	Amgen, Inc.
Title	Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement
Documents submitted	package-data.xml ep-oppo.pdf (2 p.) Published-Evidence-1.pdf\D6 - Hill .pdf (2 p.) ep-opposition-data.xml OTHER-1.pdf\AMG17203OP_Filing_D6.pdf (1 p.)
Submitted by	CN=Solveig Moré 24886
Method of submission	Online
Date and time receipt generated	14 August 2017, 12:55 (CEST)
Message Digest	86:9C:E8:B2:1C:EC:85:15:F8:22:97:8E:88:50:6C:CC:E8:5C:D1:40

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/

Samsung Bioepis Exhibit No. 1002
Page 6101

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European Patent Office
Office européen des brevets

European Patent Office
80298 MUNICH
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Hoffmann Eitle
Patent- und Rechtsanwälte PartmbB
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ALLEMAGNE

Date
17.08.2017

Reference	Application No./Patent No.
198 441 u6/ne OPPO 02	11001632.6 - 1466 / 2359834
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

Request for documents pursuant Rule 83 EPC / Request for translation pursuant Rule 3(3) EPC

In the notice of opposition/letter of 09.08.2017 reference is made to the following documents:

D2, D4, D5 and D6

You are requested to file these documents / to file a translation in one of the official languages of the EPO within a period of **two months** from notification of this communication.

If the documents are not filed in good time, the European Patent Office may decide not to take into account any arguments based on them (R. 83 EPC) / (R. 3(3) EPC).

For the Opposition Division

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Registered letter

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European Patent Office
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Date
17.08.2017

Reference	Application No./Patent No.
P2913 EP/1	11001632.6 - 1466 / 2359834
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

Europäisches Patentamt • European Patent Office • Office européen des brevets

Enclosure: Notice of opposition O2 (Hoffmann Eitle) dated 09.08.2017 with non-patent literature.
Copy of Form 2319.

Samsung Bioepis Exhibit No. 1002
Page 6103

VOSSIUS & PARTNER
Patentanwälte Rechtsanwälte mbB

EPO - Munich
102
21. Aug. 2017

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

European Patent Office

80298 M U N I C H

11 00 1632.6 / 2359834
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Amgen Inc.
Vossius Ref.: P2913 OPP(EP/1)

München, August 21, 2017
ARO/CLA

This refers to the communication of a notice of opposition dated August 10, 2017. It is requested to provide us with copies of all cited documents.

Dr. Roger Absehe
European Patent Attorney

Patentanwälte - Deutschland
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)
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Dr. Friederike Stolzenburg, Dipl.-Biol. 1)
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Dr. Jürgen Meier, Dipl.-Biol. 1)
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Paul Kretschmar, LL.M. 1) 3)
Dr. Marcus v. Welser, LL.M. 3)
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2) Japanese Patent Attorney
3) Fachanwalt für gewerblichen Rechtsschutz
4) U.S. Attorney-at-Law (New York)
5) European Patent Attorney
6) Registered U.S. Patent Attorney
7) Korean Patent Attorney
8) Abogada (Spain)
9) mit Zweigstelle in Berlin
10) Solicitor (England & Wales)
11) kein European Trademark Attorney
12) auch Patentanwalt - Deutschland

Germany: Partnerschaftsregister AG München PR 89
Switzerland: HR Firmennummer CH-270.9.001.271-9

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Samsung Bioepis Exhibit No. 1002
Page 6104

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European Patent Office
80298 MUNICH
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Questions about this communication ?
Contact Customer Services at www.epo.org/contact

Vossius & Partner
Patentanwälte Rechtsanwälte mbB
Siebertstrasse 3
81675 München
ALLEMAGNE

Date: 22-08-2017

Reference	Application No./Patent No.
P2913 EP/1	11001632.6 - 1466 / 2359834

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 OI of 14.08.2017(with c.doc D6)
- ☐ 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).

☐

For the Opposition Division

Europäisches Patentamt • European Patent Office • Office européen des brevets

☐ **Registered letter**

Samsung Bioepis Exhibit No. 1002
Page 6105

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Questions about this communication ?
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Vossius & Partner
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81675 München
ALLEMAGNE

Date: 30-08-2017

Reference	Application No./Patent No.
P2913 EP/1	11001632.6 - 1466 / 2359834

Applicant/Proprietor: Alexion Pharmaceuticals, Inc.

BRIEF COMMUNICATION

Subject:
- [X] Your letter of 21.08.2017
- [ ] Our telephone conversation of
- [ ] Communication of
- [ ]

Enclosure(s):
- [ ] Letter from the proprietor of the patent of
- [ ] Letter from the opponent of
- [X] Copy (copies) Cited documents

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**

Samsung Bioepis Exhibit No. 1002
Page 6106

Europäisches Patentamt
European Patent Office
Office européen des brevets

European Patent Office
80298 MUNICH
GERMANY

Questions about this communication ?
Contact Customer Services at www.epo.org/contact

Hoffmann Eitle
Patent- und Rechtsanwälte PartmbB
Arabellastrasse 30
81925 München
ALLEMAGNE

Date
13.09.2017

Reference	Application No./Patent No.
198 441 u6/ne OPPO 02	11001632.6 - 1466 / 2359834
Applicant/Proprietor: Alexion Pharmaceuticals, Inc.	

Communication of further notices of opposition pursuant to Rule 79(2) EPC

You are hereby informed pursuant to Rule 79(2) EPC of further notices of opposition, which are also available via the European Patent Register at **www.epo.org/register**. Should you wish to receive any patent 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).

If oral proceedings are to take place, parties are advised to check the electronic file via the European Patent Register in advance of the hearing to ensure they are in possession of all relevant documents.

A copy of the communication pursuant to Rule 79(1) EPC sent to the proprietor of the patent is also enclosed for your information.

The observations of the patent proprietor on the notice of opposition will be communicated to the opponents 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 continued. Your attention is drawn to Article 114(2) EPC.

For the Opposition Division

Europäisches Patentamt • European Patent Office • Office européen des brevets

Enclosures: copy of the communication pursuant to Rule 79(1) EPC (Form 2317A)

O1 : Amgen inc. with cited documents – non patent literature –.

Samsung Bioepis Exhibit No. 1002
Page 6107

Europäisches Patentamt
European Patent Office
Office européen des brevets

European Patent Office
80298 MUNICH
GERMANY

Questions about this communication ?
Contact Customer Services at www.epo.org/contact

Vossius & Partner
Patentanwälte Rechtsanwälte mbB
Siebertstrasse 3
81675 München
ALLEMAGNE

Date
13.09.2017

Reference	Application No./Patent No.
P2913 EP/1	11001632.6 - 1466 / 2359834
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

Communication of notices of opposition (R. 79(1) EPC)

Notices of opposition have been filed within the opposition period by:

01.
Amgen Inc.
One Amgen Center Drive
Thousand Oaks, CA 91320-1799
ETATS-UNIS D'AMERIQUE
02.
Hoffmann Eitle
Patent- und Rechtsanwälte PartmbB
Arabellastrasse 30
81925 München
ALLEMAGNE

The notices of opposition indicated above have 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

Samsung Bioepis Exhibit No. 1002
Page 6108

Europäisches Patentamt
European Patent Office
Office européen des brevets

Notice of opposition to a European patent

I. Patent opposed

Patent No.	EP2359834
Application No.	EP11001632.6
Date of mention of the grant in the European Patent Bulletin (Art. 97(3), Art. 99(1) EPC)	09 November 2016
Title of the invention	Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement

II. Proprietor of the patent

first named in the patent specification	ALEXION PHARMACEUTICALS, INC.
Opponent's or representative's reference	AMG17203OP

III. Opponent

Name	Amgen Inc.
Address:	One Amgen Center Drive Thousand Oaks CA California 91320-1799 United States of America
State of residence or of principal place of business	United States of America
Multiple opponents (see additional sheet)	☐

IV. Authorisation

1. Representative

	Dörries, Mr H. Ulrich
Company:	df-mp
Address of place of business	Fünf Höfe Theatinerstraße 16 80333 Munich Germany
Telephone/Fax	+49 89 2102960 / +49 89 21029633
E-mail:	info@df-mp.com

Multiple representatives (see additional sheet) ☐

Authorisation(s)

is/are enclosed ☐

has/have been registered under No. ☐

V. Opposition is filed against

the patent as a whole ☒

claim(s) No(s).

VI. Grounds for opposition:

Opposition is based on the following grounds:

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

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

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

• patentability is excluded on other grounds, namely articles ☐

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

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

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

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

VIII. Other requests:

IX. Evidence presented

A1	Patent document	EP2359834 (A1) original file name: A1_EP 2 359 834 A1.pdf attached as: Published-Evidence-3.pdf
D1	Patent document	US2005/0191298 (A1) particular relevance: Bell, Leonard et al.; September 1, 2005 original file name: D1.pdf attached as: Published-Evidence-18.pdf
D10	Non-patent literature - internet	U.S. Pharmacopeia, "Customized plastic packaging - applied to every need" , [cited 17.05.2017] Available from: [http://www.pharmacopeia.cn/v29240/usp29nf24s0_c1136.html] original file name: D10.pdf attached as: Published-Evidence-4.pdf
D11	Other evidence	Adis Data information, Drugs R. D. 2007, 8(1), 61-68 original file name: D11.pdf attached as: Other-evidence-1.pdf
D12	Non-patent literature - article	Hill, Anita et al., "Sustained response and long-term safety of eculizumab in paroxysmal nocturnal hemoglobinuria" Bloodjournal, Vol. 106, No. 7, 2007 particular relevance: 2559-2565 original file name: D12.pdf attached as: Published-Evidence-5.pdf
D13	Non-patent literature - article	Thomas, Thomas C. et al., "INHIBITION OF COMPLEMENT ACTIVITY VY HUMANIZED ANTI-C5 ANTIBODY AND SINGLE-CHAIN Fv." Moleculary Immunology, Vol. 33, No. 17/18, 1996 particular relevance: 1389-1401 original file name: D13.pdf attached as: Published-Evidence-6.pdf
D14	Non-patent literature - article	Mueller, John P. et al., "HUMANIZED PORCINE VCAM-SPECIFIC MONOCLONAL ANTIBODIES WITH CHIMERIC IgG2/G4 CONSTANT REGIONS BLOCK HUMAN LEUKOCYTE BINDING TO PORCINE ENDOTHELIAL CELLS" Molecular Immunology, Vol. 34, No. 6, 1997 particular relevance: 441-452 original file name: D14.pdf attached as: Published-Evidence-7.pdf
D15	Patent document	WO97/11971 (A1) particular relevance: Mueller, John et. al.; April 3, 1997 original file name: D15.pdf attached as: Published-Evidence-8.pdf
D16	Patent document	WO2005/007809 (A2) particular relevance: Rother, Russell et al., January 27, 2005 original file name: D16.pdf attached as: Published-Evidence-9.pdf
D17	Patent document	US6,355,245 (B1) particular relevance: Evans, Mark J. et al., March 12, 2002 original file name: D17.pdf attached as: Published-Evidence-10.pdf

D18	Non-patent literature - article	Hillmen, M.B., Ch.N., PhD., Peter et al., "The Complement Inhibitor Eculizumab in Paroxysmal Nocturnal Hemoglobinuria" THE NEW ENGLAND JOURNAL OF MEDICINE, 2006 original file name: D18.pdf attached as: Published-Evidence-11.pdf
D19	Other evidence	EMEA, CPMP, 20 February 2003, Note for guidance on excipients original file name: D19.pdf attached as: Other-evidence-2.pdf
D2	Non-patent literature - article	Hillmen, P. et al., "Oral Communication; Presidents Free Communications 257-280" Br. J. Hematol., Vol. 121, 2003 particular relevance: (Suppl. 1), 87-95 original file name: D2.pdf attached as: Published-Evidence-19.pdf
D20	Non-patent literature - article	Wei, Wang et al., "Anitbody Structure, Instability, and Formulation" Journal of Pharmaceutical Sciences, Vol. 96, No. 1, January 2007 original file name: D20.pdf attached as: Published-Evidence-12.pdf
D21	Non-patent literature - article	Daugherty, Ann L. et al., "Formulation and delivery issues for monoclonal antibody therapeutics" ScienceDirect - Advanced Drug Delivery Reviews, Vol. 58, May 2006 particular relevance: 686-706 original file name: D21.pdf attached as: Published-Evidence-13.pdf
D22	Patent document	US2005/0271660 (A1) particular relevance: Wang, Yi; December 8, 2005 original file name: D22.pdf attached as: Published-Evidence-14.pdf
D23	Other evidence	Expert declaration by Dr. Michael Treuheit; CV; Appendix A-C original file name: D23.pdf attached as: Other-evidence-3.pdf
D24	Other evidence	Soliris® (eculizumab) Highlights of prescribing information original file name: D24.pdf attached as: Other-evidence-4.pdf
D25	Patent document	US2012/0230982 (A1) particular relevance: Zhou, Xiao-Hong et al., September 13, 2012 original file name: D25.pdf attached as: Published-Evidence-15.pdf
D26	Patent document	WO2007/002571 (A2) particular relevance: Rother, January 4, 2007 original file name: D26.pdf attached as: Published-Evidence-16.pdf
D27	Non-patent literature - article	Hill, Anita et al., "Sustained Control of Hemolysis and Symptoms and Reduced Transfusion Requirements over a Period of 2 Years in Paroxysmal Nocturnal Hemoglobinuria (PNH) with Eculizumab Therapy" Blood, Vol. 104, 2004 particular relevance: 2823-2824 original file name: D27.pdf

		attached as: Published-Evidence-17.pdf
D3	Non-patent literature - article	Hillmen, M.B., Ph.D., Peter et al., "Effect of Eculizumab on Hemolysis and Transfusion Requirements in Patients with Paroxysmal Nocturnal Hemoglobinuria" THE NEW ENGLAND JOURNAL OF MEDICINE, Vol. 350, No. 6, 2004 particular relevance: 552-559 original file name: D3.pdf attached as: Published-Evidence-20.pdf
D4	Non-patent literature - article	Harding et al., "Eculizumab - Huminazed Anti-C5 Monoclonal Antibody" Drugs of the Future, Vol. 29, No. 7, 2004 particular relevance: 673-676 original file name: D4.pdf attached as: Published-Evidence-21.pdf
D5	Patent document	US2005/0221382 (A1) particular relevance: Rother, P. Russell; October 6, 2005 original file name: D5.pdf attached as: Published-Evidence-22.pdf
D7	Non-patent literature - article	Morris et al., "Recurrence of neisserial meningococcemia due to deficiency of terminal complement component" Southern medical journal, Vol. 25, No. 19, 1992 ISSN: 1541-8243 particular relevance: 1030-1031 original file name: D7.pdf attached as: Published-Evidence-23.pdf
D8	Non-patent literature - article	Corvini MD, Michael et al., "Complement C7 deficiency presenting as recurrent aseptic meningitis" ANNALS OF ALLERGY, ASTHMA & IMMUNOLOGY, Vol. 93, No. 2, 2004 particular relevance: 200-205 original file name: D8.pdf attached as: Published-Evidence-24.pdf
D9	Non-patent literature - article	GVG, Sierra et al., "Vaccine Against Group B Neisseria Meningitidis: Protection Trial And Mass Caccination Results In Cuba" Niph Annals, Vol. 14, No. 2, 1991 ISSN: 0332-5652 original file name: D9.pdf attached as: Published-Evidence-25.pdf
P	Patent document	US2006/0783070 P (P) particular relevance: Bell, Leonard et. al.; March 15, 2006 original file name: P.pdf attached as: Published-Evidence-1.pdf
WO	Patent document	WO2007/106585 (A1) original file name: WO.pdf attached as: Published-Evidence-2.pdf

X. Payment

Mode of payment	Debit from deposit account

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

Currency:	EUR
Deposit account number:	28001000
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Any refunds should be made to EPO deposit account:	28001000
Account holder:	df-mp

Fees	Factor applied	Fee schedule	Amount to be paid
010 Opposition fee	1	785.00	785.00
Total:		EUR	785.00

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

	B Attached document files	Original file name:	System file name:
B-1	1. Facts and arguments	AMG17203OP_Notice of Opposition.pdf	OPPO.pdf

	C Attached evidence files	Original file name:	System file name:
C-1	1. Patent document	P.pdf	Published-Evidence-1.pdf
C-2	2. Patent document	WO.pdf	Published-Evidence-2.pdf
C-3	3. Patent document	A1_EP 2 359 834 A1.pdf	Published-Evidence-3.pdf
C-4	4. Patent document	D15.pdf	Published-Evidence-8.pdf
C-5	5. Patent document	D16.pdf	Published-Evidence-9.pdf
C-6	6. Patent document	D17.pdf	Published-Evidence-10.pdf
C-7	7. Patent document	D22.pdf	Published-Evidence-14.pdf

C-8	8. Patent document	D25.pdf	Published-Evidence-15.pdf
C-9	9. Patent document	D26.pdf	Published-Evidence-16.pdf
C-10	10. Patent document	D1.pdf	Published-Evidence-18.pdf
C-11	11. Patent document	D5.pdf	Published-Evidence-22.pdf
C-12	1. Non-patent literature - article	D12.pdf	Published-Evidence-5.pdf
C-13	2. Non-patent literature - article	D13.pdf	Published-Evidence-6.pdf
C-14	3. Non-patent literature - article	D14.pdf	Published-Evidence-7.pdf
C-15	4. Non-patent literature - article	D18.pdf	Published-Evidence-11.pdf
C-16	5. Non-patent literature - article	D20.pdf	Published-Evidence-12.pdf
C-17	6. Non-patent literature - article	D21.pdf	Published-Evidence-13.pdf
C-18	7. Non-patent literature - article	D27.pdf	Published-Evidence-17.pdf
C-19	8. Non-patent literature - article	D2.pdf	Published-Evidence-19.pdf
C-20	9. Non-patent literature - article	D3.pdf	Published-Evidence-20.pdf
C-21	10. Non-patent literature - article	D4.pdf	Published-Evidence-21.pdf
C-22	11. Non-patent literature - article	D7.pdf	Published-Evidence-23.pdf
C-23	12. Non-patent literature - article	D8.pdf	Published-Evidence-24.pdf
C-24	13. Non-patent literature - article	D9.pdf	Published-Evidence-25.pdf
C-25	14. Non-patent literature - internet	D10.pdf	Published-Evidence-4.pdf
C-26	1. Other evidence	D11.pdf	Other-evidence-1.pdf
C-27	2. Other evidence	D19.pdf	Other-evidence-2.pdf
C-28	3. Other evidence	D24.pdf	Other-evidence-4.pdf
C-29	4. Other evidence	D23.pdf	Other-evidence-3.pdf

Signature of opponent or representative

Place	**Munich, Germany**
Date:	**04 August 2017**
Signed by:	**Hans Doerries 13689**
Representative name:	**H. Ulrich Dörries**
Capacity:	**(Representative)**

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August 4, 2017

Opposition against European patent 2 359 834 (Application No. 11 001 632.6)

Title:	Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement
Proprietor:	Alexion Pharmaceuticals, Inc.
Opponent:	Amgen Inc.
Our Ref.:	AMG17203OP

On behalf of Amgen Inc.
One Amgen Center Drive
Thousand Oaks, CA 91320-1799
USA

we herewith file a **Notice of Opposition** pursuant to Article 99(1) EPC against

European Patent No. 2 359 834 B1
(European Appl. No. 11 001 632.6)

Publication and mention of the grant: 09.11.2016

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

Proprietor: Alexion Pharmaceuticals Inc.
100 College Street
New Haven, CT 06510 / US

We request that the opposition fee in the amount of EUR 785.00 be debited from our deposit account No. 28001000 (see also the corresponding debit order on the form accompanying the online submission of the present Notice of Opposition).

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8 Rechtsanwalt

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I. Extent of the Opposition and Requests

1. The opposition is directed against the (sole) claim of European patent 2 359 834 B1 (hereinafter referred to as "the opposed patent"). It is requested that the opposed patent be revoked in its entirety pursuant to Article 101(2) EPC.

2. Oral Proceedings are requested in accordance with Article 116(1) EPC in the event that the Opposition Division does not consider the above request justified on the basis of our written submissions.

II. Grounds in Support of the Opposition

3. The opposition is based on the following grounds:

   i) The claimed subject matter of the opposed patent extends beyond the content of the earlier application as filed (Art. 100(c) EPC in connection with Art. 76(1) EPC, 123(2) EPC).
   ii) The subject matter of the opposed patent is not based on an inventive step (Art. 100(a) EPC in connection with Art. 56 EPC).
   iii) The subject matter of the opposed patent is insufficiently disclosed (Art. 100(b) EPC).

III. List of Documents

4. The following documents were cited during the prosecution of the patent EP 2 359 834 B1, hereinafter "the opposed patent"; they are enclosed herewith except for **D6** which we were unable to retrieve:

D1 US 2005/191298 A1 (Bell, Leonard *et al.*; September 1, 2005).

D2 Hillmen *et al.*, *Br. J. Hematol.* **2003**, *121 (Suppl. 1)*, 87-95.

D3 Hillmen *et al.*, *N. Engl. J. Med.* **2004**, *350(6)*, 552-559.

D4 Harding, *Drugs Future* **2004**, *29(7)*, 673-676.

D5 US 2005/221382 A1 (Rother, P. Russell; October 6, 2005)

D6 Hill, *Clin. Adv. Hematol. Oncol.* **2005**, *3(11)*, 849-50.

D7 Morris et al., *South. Med. J.* **1992**, *85(19)*, 1030-1031.

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D8 Corvini *et al.*, *Ann. Allerg. Asth. Immunol.* **2004**, *93(2)*, 200-205.

D9 Sierra *et al.*, *NIPH Annals* **1991**, *14(2)*, 195-210.

5. The following additional documents are also referred to herein and submitted herewith:

P US 2006/0783070 P (Bell, Leonard *et al.*; March 15, 2006).

WO WO 2007/106585 A1.

A1 EP 2 359 834 A1.

D10 US Pharmacopeia (USP29-NF24), p. 2989;
retrieved from http://www.pharmacopeia.cn/v29240/usp29nf24s0_c1136.html
on May 17, 2017.

D11 Adis Data information, *Drugs R. D.* **2007**, *8(1)*, 61-68.

D12 Hill *et al.*, *Blood* **2005**, *106(7)*, 2559-2565.

D13 Thomas *et al.*, *Mol. Immunol.* **1996**, *33(17/18)*, 1389-1401.

D14 Mueller *et al.*, *Mol. Immunol.* **1997**, *34(6)*, 441-452.

D15 WO 97/11971 A1 (Mueller, John *et al.*, April 3, 1997).

D16 WO 2005/007809 A2 (Rother, Russell *et al.*, January 27, 2005).

D17 US 6,355,245 B1 (Evans, Mark J. *et al.*, March 12, 2002).

D18 Hillmen *et al.*, *N. Engl. J. Med.* **2006**, *355(12)*, 1233-1243.

D19 EMEA, CPMP, 20 February 2003, *Note for guidance on excipients, antioxidants and antimicrobial preservatives in the dossier for application for marketing authorization of a medicinal product*.

D20 Wang *et al.*, *J. Pharm. Sci.* **2007**, *96(1)*, 1-26. (published January 2007).

D21 Daugherty *et al.*, *Adv. Drug Del. Rev.* **2006**, *58*, 686-706.

D22 US 2005/0271660 A1 (Wang, Yi; December 8, 2005).

D23 Expert declaration by Dr. Michael Treuheit.

D24 Soliris® (eculizumab) *Highlights of prescribing information*.

D25 US 2012/0230982 A1 (Zhou, Xiao-Hong *et al.*, September 13, 2012).

D26 WO 2007/002571 A2 (Rother, January 4, 2007).

D27 Hill *et al.*, *Blood* **2004**, *104*;2823-2824.

IV. The Claimed Subject Matter

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

V. Inadmissible Amendment (Art. 100 (c) EPC / Art. 76 (1) EPC, 123(2) EPC)

7. The parent application as filed (**WO**), only teaches a pharmaceutical composition for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein the composition is a 300 mg eculizumab single-use dosage form comprising 30 ml of a 10 mg eculizumab/ml sterile, preservative free solution in the context of a *single unit dosage form*, both on page 5, lines 3-9 and page 22, lines 7-14.

8. The same applies for the corresponding passages of the divisional application **A1**, which also require a single unit dosage form (paragraphs [0018] and [0024)]. This understanding is confirmed by claim 1 of **A1**, which recites: *"[a] pharmaceutical composition comprising* ***a single unit dosage form*** *of an anti-C5 antibody"* (emphasis added).

9. It is noted in this context that the skilled person would understand a single-use dosage form to be for single use only, i.e., it cannot be used more than once. In contrast, a single unit dosage form would be understood to be synonymous to a single-unit container, which, as evidenced by the US Pharmacopeia (submitted as **D10** herewith), "*is designed to hold a quantity of drug product intended for administration as a single dose or a single finished device intended for use promptly after the container is opened*".

10. As an essential element present in the parent application as filed as well as the divisional application as filed, namely, the requirement for administration of the 300 mg dose as a single dose is missing from the claim as granted, the claim is not clearly and unambiguously derivable from the application as filed, contrary to Art. 76(1) EPC.

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VI. No Entitlement to Priority

11. The opposed application claims priority of US provisional application US 783070 P (**P**). Said application does not contain any disclosure of a pharmaceutical composition comprising 300 mg of eculizumab in a volume of 30 mL, at a concentration of 10 mg/mL in a single-use dosage form of a sterile, preservative-free solution. None of these specific details, let alone their combination, is disclosed in **P**.

12. The claim of the opposed patent thus is not entitled to priority. Consequently, all documents available before the **filing date of March 15, 2007** are considered relevant state of the art.

VII. Lack of Inventive Step (Article 100(a) EPC / Article 56 EPC)

VII.1 The state of the art

13. Eculizumab, also referred to as h5G1.1 (**D11**, abstract; **D4**, abstract; **D1**, [0052]); opposed patent [0054]), is a long-acting, humanized version of the anti-C5 antibody 5G1.1 that has been under development as a therapeutic for a range of different indications since 1995 (**D11**). Indications known before the filing date of the opposed patent include rheumatic disorders, transplant rejection, adult respiratory distress syndrome, lupus nephritis and systemic lupus erythematosus, glomerulonephritis, psoriasis, dermatomyositis, pulmonary diseases, sickle cell disease, and also paroxysmal hemoglobinuria (PNH) (**D11**, Table II; **D4**, abstract and "Clinical Studies" on pages 674-675; **D22**, claims 1, 2, and 7; **D26**, e.g., claims 1 and 14; **D5** abstract, par. [0027] and [0040]).

14. The complement cascade is a part of the human immune system that binds and removes foreign particles, but can also attack and destroy endogenous cells (such as red blood cells) or tissue. The antibody eculizumab has been developed to inhibit the terminal steps of the complement cascade, in particular, eculizumab suppresses the cleavage of complement component 5 (or C5) into C5a and C5b (**D12**, p. 2559, right column, **D4**, page 673, right column, 2nd full paragraph).

15. The opposed patent at [0054] states that eculizumab is disclosed in **D17**. The variable heavy and light chain regions of eculizumab/h5G1.1 were disclosed in **D17** in the context of antibody fragments, such as an scFv (**D17**, SEQ ID NO: 20, "5G1.1 scFv CO12 (humanized)"). This document states that preferred constant regions for forming full length antibodies would be "IgG constant regions, which may be unaltered or constructed of a mixture of constant domains from IgGs of various subtypes, e.g., IgG1 and IgG4." (**D17**, col. 45, li. 24-33).

16. The first humanized 5G1.1 antibody (h5G1.1) was published in 1996 (**D13**, p. 1392, Fig. 1 A and B).

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17. An advantageous combination of h5G1.1, and in particular h5G1.1 CO12, with chimeric human IgG2/IgG4 heavy chain constant regions is shown and/or suggested in **D14, D15** and **D16**: **D14** shows optimization of the antibody by the introduction of a chimeric human IgG2/IgG4 heavy chain constant region (**D14**, Fig. 7, Fig. 9, and paragraph bridging pages 450/451 and 2nd paragraph on p. 451; see also **D15**, e.g., p. 42-43; and **D16**, Fig. 2 and claim 3), which was shown to advantageously avoid undesired immune responses, FcR binding and complement activation (**D14**, paragraph bridging pages 450/451; **D15**, Fig. 15 and p. 12, li. 37; **D16,** Fig. 2, paragraph bridging pages 5 and 6).

18. The mechanism of action of eculizumab and preferred antibody structures were thus known prior to the filing date of the opposed patent, and eculizumab had been used as a therapeutic agent for a number of indications aside from PNH.

19. PNH is a hematologic disorder, symptomatically characterized by red blood cell destruction, aenemia, hemoglobinuria and thrombosis. The symptoms result from somatically mutated hematopoietic blood cells, causing a deficiency of glycosylphosphatidylinositol (GPI) anchored cell surface proteins. Among these proteins are complement inhibitors that normally protect red blood cells from complement-mediated lysis (**D12**).

20. The treatment of patients suffering from PNH with anti-C5 antibodies, such as eculizumab or pexelizumab was also widely known before the filing date. In the case of PNH, the blockade of the terminal steps of the complement cascade by eculizumab serves to block the undesired destruction of red blood cells; however, it preserves the ability of the complement system to clear microorganisms and immune complexes (**D12**, p. 2559, right column).

21. A treatment with eculizumab in a dosage of 600 mg over the first four weeks, followed by an administration of 900 mg as the fifth dose one week later, and subsequent administration of 900 mg on a bi-weekly basis is disclosed, e.g., in **D1** (par. [0082]). The route of administration was intravenous infusion (ibid.). This corresponds exactly to the dosage regimen described in the opposed patent ([0057]).

22. Further successful clinical trials of eculizumab are described in detail, in e.g. **D2** (in 2003), **D3** (in 2004), **D4** (in 2004), **D12** (in 2005), **D18** (in 2006) and **D11** (in 2007). **D27** (from 2004, abstract) teaches that treatment of PNH by eculizumab has been shown to be safe and effective for over two years of therapy. Dosage regimens requiring administration of both 600 and 900 mg eculizumab for treatment of PNH are explicitly disclosed in **D2** (li. 7 and 8), **D3** (Abstract), **D4** (page 674, last full paragraph), **D11** (last paragraph on page 66) and **D18** (page 1233, Methods), so they can be considered to be conventional.

23. **D18** in fact discloses the clinical trial that serves as the sole example in the opposed patent (the TRIUMPH trial). This includes the vaccination of all treated patients against *Neisseria meningitides* (**D18**, p. 1234, right column, 1st full paragraph), a feature that was brought forward by the applicant in the course of the prosecution in an attempt to distinguish the alleged invention from the state of the art, and later dropped again. The vaccination serves to

avoid side effects of the inhibition of complement formation in the treated patients leading to a higher susceptibility to *Neisseria* infections. This problem and its solution were well known in the art, as pointed out by the examiner with reference to **D6**, **D7** and **D8**.

24. The skilled person was consequently aware of suitable formulations for the intravenous administration of eculizumab, and these had already been successfully used in several clinical trials for PNH, as well as for treatment of numerous other diseases.

VII.2 *The closest prior art*

25. **D1** – or any of the reports of intravenous treatment of PNH with eculizumab at doses of 600 or 900 mg/ml, i.e., **D2** to **D4**, **D11** and **D18** - can be considered the closest prior art document, as all of them are highly promising springboards for the claimed subject matter.

26. We submit that it is an implicit feature of all compositions for intravenous administration that they are sterile. **D1** not only discloses the treatment of PNH with eculizumab and dosing regimen, in particular doses of 600 and 900 mg in par. [0082], as mentioned above, but also explicitly teaches that suitable formulations

> *"[…]* ***must be sterile*** *and non-pyrogenic, and generally will include a pharmaceutically effective carrier, such as saline, buffered (e.g., phosphate buffered) saline, Hank's solution, Ringer's solution, dextrose/saline, glucose solutions, and the like. The formulations* ***may contain*** *pharmaceutically acceptable auxiliary substances as required, such as, tonicity adjusting agents, wetting agents, bactericidal agents,* ***preservatives****, stabilizers, and the like."* (**D1**, par. [0062], emphasis added)

D1 thus also teaches that in contrast to the strict requirement for sterility, the presence of preservatives is optional. We thus suggest **D1** as the closest prior art document.

VII.3 *The subject matter of the claim*

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

28. **D1** does not explicitly teach that eculizumab is in a single-use dosage form of 300 mg comprising 30 ml of a concentration of 10 mg/ml, or that the composition is preservative free. It is noted that the amount of antibody and the concentration thereof define the volume of the composition, which consequently cannot be considered an independent technical feature.

VII.4 The technical effect and the problem solved

29. Notably, the opposed patent is silent about supposed technical effects or advantages of any one of these features.

30. As the treatment of patients suffering from PNH with eculizumab was well known, and a suitable pharmaceutical formulation for the antibody thus clearly already existed prior to the filing of the opposed patent, it is not apparent which technical problem is solved by the alleged invention.

31. Submissions by the applicant in the communications dated February 6, 2016 (p. 6, item 3.2) and January 13, 2016 (p. 5, item 4.3.1) imply that the technical problem to be solved was the provision of a stable and easy-to-use solution of eculizumab. This is nowhere mentioned in the opposed patent itself. The only evidence brought forward by the applicant with regard to the alleged technical effect is the package leaflet of Soliris® (eculizumab). This is supposed to prove that the marketed concentration is in fact the "*ideal and clinically approved*" concentration for the antibody (communication dated January 13, 2016, item 4.3.1). While it obviously is the clinically approved concentration, this does not represent evidence for 10 mg/mL being the "ideal" concentration. It merely shows that formulating eculizumab at 10 mg/mL is feasible. Accordingly, there is no evidence for an allegedly ideal increased stability.

32. **D1** does not teach if the administered composition comprises preservatives.

33. **D19**, a note for guidance to industry from EMEA, which can be considered to represent common general knowledge, states that the properties of antioxidants and antimicrobial preservatives are due to certain chemical groups which are usually aggressive towards living cells and which lead to certain risks when used in man. Thus, if it is not absolutely necessary to add these substances in medicinal products, they must be avoided (**D19**, page 1/9, paragraphs 4-6 of introduction).

34. Accordingly, the fact that the claimed compositions do not contain preservatives has the technical effect that potential issues connected to the use of preservatives, both from the medical and the regulatory viewpoint, are avoided.

35. Consequently, the problem solved by the invention can be considered to be provision of a composition which avoids potential medical and/or the regulatory issues connected to the use of preservatives.

VII.5 *The subject matter of the claim lacks inventive step in light of **D1** in combination with **D22** and common general knowledge, represented by **D19** and **D20-D21**.*

36. It has to be noted that the opposed patent teaches no advantageous effect of not using **preservatives**. In contrast, it states that among other excipients, preservatives can be present in the pharmaceutical compositions of the alleged invention (par. [0058] and [0064]).

37. Nevertheless, the skilled person who intends to produce eculizumab compositions for therapeutic application, as in **D1** (or in the opposed patent), is motivated to exclude preservatives for regulatory reasons. The German Bundesgerichtshof in "Anthocyanverbindung" (X ZR 4/11, 10.12.2013) recently confirmed that legal (including regulatory) considerations are to be taken into account by the skilled person:

> "*The skilled person to which provision of a compound for a certain application is assigned has occasion to check according to the relevant legal provisions which solutions have adequate prospects of success with regard to legal aspects*" (headnote; our translation).

38. **D19**, as cited above, not only provides such a relevant legal provision, i.e., a motivation to avoid preservatives (**D19**, page 1/9, paragraphs 4-6 of introduction), but also teaches that multi-dose preparations are most problematic with regard to the risk of contamination by micro-organisms (pages 8-9/9, section "Antimicrobial Preservatives", In the last sentence on page 9/9, the document clearly teaches:

> "*If products do not contain a preservative and do not have inherent preservative efficacy they must not be packaged in multidose presentations without a sound justification.*"

39. Accordingly, **D19**, which we submit is representative of common general knowledge, can be summarized to teach away from the use of preservatives and, accordingly, multidose presentations. Following this clear teaching, the skilled person would turn to **single-use** packaging to avoid the requirement for preservatives.

40. The skilled person would also not have expected any difficulties with this approach, as it was well known to the person of skill that antibody formulations for intravenous delivery can be prepared without using preservatives, as **D20** (p. 2-4, Table 1) and **D21** (p. 697, Table 1) show. In fact, the majority of antibody formulations reviewed in these documents do not contain preservatives. In addition, **D22** specifically discloses a formulation of eculizumab that does not contain preservatives (**D22**, Fig. 10). As a consequence, the person of skill would have readily assumed, based on the state of the art, that a single-use pharmaceutical formulation comprising eculizumab would not require the use of preservatives.

41. The skilled person was consequently faced with the selection of an appropriate **amount of antibody** to include in the single-use package.

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42. As discussed above, e.g., **D1** (par. [0082]) teaches that the administration of either 600 mg, or 900 mg is the established dosing regimen for eculizumab. In full agreement with the examiner (communication dated July 21, 2011), we note that 300 mg is the obvious and most convenient single dose that enables supplying both therapeutic doses of 600 and 900 mg, while avoiding a waste of active agent. We note that the applicant did not argue that there is any unexpected technical effect or advantage associated with this feature. The amount of antibody included is thus rendered obvious by **D1** itself.

43. This leaves the antibody **concentration of 10 mg/mL** as a distinguishing feature. The applicant in his communications dated February 6, 2014 and January 13, 2016 alleged that the concentration was an inventive feature:

> *"At a minimum, in order to arrive at the instantly-claimed dosage forms at all, the inventors had to know that eculizumab was soluble. In order to produce a solution of an antibody (e.g., at 10 mg/mL), the inventors had to know the solubility of eculizumab and that eculizumab was sufficiently soluble for a 10 mg/mL formulation. Moreover, in order for a pharmaceutical solution to be commercially viable, the inventors had to know the shelf-life stability of eculizumab in solution; for if its stability was too low, a pharmaceutical solution would not have been an option. Thus, even assuming arguendo that the instantly claimed compositions are convenient, it is clear that the skilled person reading the cited references could not have arrived at the particular instantly claimed compositions by convenience. They would have required the knowledge supporting the instant invention. And none of that knowledge is disclosed or even suggested by the cited references."* (Communication submitted by applicant on February 6, 2014, p. 6, item 3.2, emphasis added)

and

> *"As indicated in section 3.2 of our petition of February 6, 2014 it was to be established at which concentration eculizumab can be solved and it was found that 10 mg eculizumab can solved in 1 ml of a sterile, preservative free solution. In addition it was to be determined whether eculizumab is storage-stable at a concentration of 10 mg/ml."* (Communication submitted by applicant on January 13, 2016, p. 5, item 4.3.1, emphasis added)

44. In contrast, we submit that the concentration of 10 mg/ml is an arbitrary choice made by the skilled person in the context of routine experimentation. Determining solubility and stability furthermore is part of routine experimentation in protein formulation and does not involve any inventive activity.

45. In a highly similar case, concerned with liquid stable protein formulations (**T 1241/03**), the Board of Appeals ruled that despite the understandable uncertainties, which are associated

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with developing liquid stable protein formulations, obviousness is not only at hand when the results are clearly predictable, but also when there is a reasonable expectation of success (**T 149/93**). This does not require certainty (**T 338/97**). Even in the absence of a reasonable expectation of success, the person of skill would adopt a "try and see" attitude (**T 333/97**).

46. **D5**, a document concerned with use of eculizumab for treatment of rheumatoid arthritis, filed by a co-inventor of the opposed patent, already teaches that possible formulations for complement inhibiting antibodies such as 5G1.1-mAb (claim 4), including those for intravenous infusion, that *"may conveniently be presented in unit-dosage form [and] may be prepared according to conventional techniques well known in the pharmaceutical industry"* (**D5**, par. [0043]). Paragraph [0046] similarly refers the skilled person to guidance in the literature and common general knowledge regarding particular dosages and methods of delivery. This confirms that the skilled person did not consider formulation of eculizumab as requiring anything more than common general knowledge and routine experimentation.

47. We agree with **D5** that there certainly was guidance in the prior art for comparable antibody formulations, e.g., **D20**, **D21** and **D22,** so that the skilled person had good reasons to assume that 10 mg/mL would represent a suitable concentration. The review articles **D20** (p. 2-4, Table 1) and **D21** (p. 697, Table 1) show that it was common general knowledge that 10 mg/mL was a common concentration for antibody formulations for intravenous delivery. The formulations listed in these documents comprise antibody concentrations ranging from 0.25 mg/mL to 100 mg/mL for intravenous (IV) delivery. **D21** lists 15 different antibody formulations for IV delivery (Table 1), wherein 10 mg/mL is one of the three most common. **D20** lists 18 formulations for IV delivery, of which 10 mg/mL is the most common (Table 1, entries 3, 14, 16 and 20).

48. At the same time, **D22** indicates that the very same antibody as presently claimed, eculizumab, can be formulated in concentrations ranging from 1-30 mg/mL (**D22**, Fig. 10 and par. [0170] to [0172]). A formulation of eculizumab at 30 mg/mL is shown to be suitable for nebulization. While **D22** is concerned with antibody formulations for a different route of delivery, this does not affect the need for both stability and solubility of the antibody. Based on **D22**, there would thus have been no uncertainties left for the skilled person that 10 mg/mL were stable and soluble, and thus a suitable concentration. At least, the formulation disclosed in said document would have provided the skilled person with a reasonable expectation of success when formulating eculizumab at 10 mg/mL.

49. We furthermore submit that the specific concentration of 10 mg/mL is an arbitrary choice, and, consequently, this feature cannot contribute to inventiveness, as established in **T 206/91** (item 5.5) and **T 37/82** (item 3). We herewith provide the results of a study of the solubility and the long-term stability of eculizumab at various concentrations in the form of a declaration submitted as **D23**.

50. The eculizumab used in the study described in **D23** was recombinantly expressed. The sequence of the heavy and light chains were confirmed to be correct by comparison to peptide

mapping of commercially acquired eculizumab in Soliris® and to the sequences provided within publically available documents the patentee filed with government agencies.

51. For the analysis, the antibody was dissolved in phosphate buffered saline (pH = 7), supplemented with polysorbate 80. These buffer conditions are identical to those used for the pharmaceutical composition of the opposed patent (see prescribing information for Soliris® (eculizumab), **D24**). The stability of solutions containing 2 mg/ml, 5 mg/ml, 10 mg/mL, 30 mg/mL and 50 mg/mL was monitored over a time course of 13 weeks at storage conditions (4 °C) and at elevated temperature (25 °C). Physical and chemical stability were analyzed by size-exclusion ultra high-performance liquid chromatography (SE-UHPLC), anion exchange high-performance liquid chromatography (AEX-HPLC), and reduce sodium dodecyl sulfate capillary electrophoresis (rCE-SDS).

52. Aggregation of the antibody results in the formation of high molecular weight (HMW) species that can be visualized by SE-UHPLC. A large degree of HMW formation is a sign of low long-term stability. All of the eculizumab solutions stored at 4°C and 25°C were sufficiently stable throughout the study, i.e., there was no significant loss in main peak for any of the protein concentrations examined. (**D23**, Appendix A)

53. The storage of antibodies in solution can result in chemical degradation, leading to the formation of acidic species (e.g. by deamidation of asparagine residues), which can be detected by AEX-HPLC. A high degree of acidic species is a sign of low chemical stability. Analysis of the eculizumab solutions (**D23**, Appendix B) shows that all tested samples suffer from some chemical degradation under the tested conditions and the degradation increases over time upon storage at the elevated temperature. There is no significant difference in chemical stability between 10 mg/mL and the other concentrations.

54. rCE-SDS offers high resolution separation of most types of chemically or structurally altered species from the main (intact) species for both heavy chain (HC) and light chain (LC) of the antibody. Analysis of the eculizumab solutions by rCE-SDS (**D23**, Appendix C) shows that a detectable decrease of the main species (HC+LC) occurs at both tested temperatures for all samples. However, in agreement with the AEX-HPLC measurements, there is no significant difference across protein concentrations.

55. Taken together, this shows that solutions of eculizumab possess similar long-term stability at concentrations of 2 mg/ml, 5 mg/ml, 10 mg/mL, 30 mg/mL or 50 mg/mL. The 10 mg/mL concentration offers no apparent advantage over the other tested concentrations.

56. Document **D25** also discloses formulations of anti-C5 antibodies and specifically eculizumab (**D25**, e.g., par. [0019], [0030], [0042], [0074], Fig. 1) at concentrations ranging from 10 mg/mL to more than 200 mg/mL (**D25**, e.g., par. [0061], [0062], [0076]). Formulations of eculizumab at various concentrations were found to be stable over a period of two years (**D25**, par. [0304]). While **D25** was published after the filing date of the opposed patent, it confirms that eculizumab can be stably formulated in various buffers in a wide range of concentrations.

57. In summary, in light of **D1**,

- the skilled person was motivated to formulate eculizumab for treatment of PNH as a single-use dosage form avoiding the use of preservatives at least due to regulatory aspects and common general knowledge (as evidenced by **D19**);
- the amount of antibody of 300 mg included in the single-use dosage form is rendered obvious by **D1** itself;
- it was common general knowledge (**D20**, **D21**) that 10 mg/mL is a conventional concentration for antibody formulations for intravenous delivery; and there was a reasonable expectation of success for this concentration of eculizumab to be stable and soluble in light of **D22**; furthermore, as confirmed by **D25** and **D23**, the concentration of 10 mg/mL was an arbitrary choice.

58. Accordingly, the claimed subject matter lacks an inventive step in light of **D1** in combination with **D22** and common general knowledge (**D19**, **D20**, **D21**), contrary to Article 56 EPC.

VIII. Insufficient Disclosure (Article 100(b) EPC)

59. The opposed patent claims a pharmaceutical composition, wherein the composition is a solution of "eculizumab". While we hold that eculizumab is clearly and unambiguously disclosed by the cited prior art (e.g., **D1** to **D5**, **D11** to **D15**, **D17**, **D18**, **D22**, **D26**, **D27**), we submit that the skilled person may be confused in this regard by the information contained in the opposed patent, to the extent that the skilled person may no longer be able to carry out the teaching of the patent. In particular, the description offers divergent definitions of the antibody eculizumab and fails to provide a correct sequence of the light chain of the antibody.

60. First, SEQ ID NOs 1 to 4 of the opposed patent supposedly define the variable heavy chain (SEQ ID NO: 1) and light chain (SEQ ID NO: 3) segments and the full length heavy chain (SEQ ID NO: 2) and light chain (SEQ ID NO: 4) of eculizumab. We note that the sequence given for the full length light chain of eculizumab is not identical to the sequence of eculizumab in the post-published document **D25** (par. [0094]), which has a length of 214 amino acids. This length of 214 amino acids is in accordance with the prescribing information for Soliris® (**D24**, item 11). To the contrary, SEQ ID NO: 4 of the opposed patent contains 22 additional N-terminal amino acids and has a length of 236 amino acids.

61. It may be concluded that the antibody described in the opposed patent, comprising heavy chains with a sequence of SEQ ID NO: 2 and light chains with a sequence of SEQ ID NO: 4 is in fact not eculizumab.

62. In conclusion, the skilled person seeking to reproduce the alleged invention would be misguided by the opposed patent into using an antibody that may not be eculizumab. Use of the antibody eculizumab for treatment of PNH can thus be considered insufficiently disclosed by the opposed patent.

63. The claim of the opposed patent violates Article 83 EPC.

IX. Conclusion

64. As the subject matter of the claim of the opposed patent

- is not clearly and unambiguously derivable form the parent application as filed, contrary to Art. 76(1) EPC and Art. 123(2) EPC,
- lacks inventive step over **D1** in combination with **D22** and common general knowledge, as evidenced by **D19** and **D20**-**D21**,
- is insufficiently disclosed,

revocation of European patent EP 2 359 834 B1 in its entirety is justified.

Respectfully submitted,

Dr. H. Ulrich Dörries
European Patent Attorney

UD/MO/SI/KS

Enclosures
Documents P, WO, A1, D1-D5 to D7-D27

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D10

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〈 1136 〉 PACKAGING—UNIT-OF-USE

INTRODUCTION

This chapter provides guidance in the use and application of unit-of-use packaging and is intended for use by drug manufacturers, repackagers, and pharmacists. Suppliers of packages and packaging components may find the information useful, as well.

The *General Notices* defines a unit-of-use container as one that contains a specific quantity of a drug product that is intended to be dispensed as such without further modification except for the addition of appropriate labeling.

Unit-of-use packaging, when provided by the manufacturer, offers some of the following attractive advantages. (1) A dosage form can be dispensed to a patient in the manufacturer's original container, a practice that recognizes that the suitability of the container has been established on the basis of the manufacturer's stability studies. (2) The counting and repackaging of dosage units in the pharmacy is eliminated, thereby reducing the possibility of human error. (3) The pharmacist is able to affix the label for the patient onto the unit-of-use package and is free to use the manufacturer's expiration date as the beyond-use date. (4) The number of dosage units in a single unit-of-use package may be determined on a case-by-case basis. (5) Patient compliance is improved. (6) The unit-of use package can protect against countertfeiting because traceability of product is ensured through bar coding techniques and NDC numbers.

Unit-of-use packaging, when provided by repackagers, offers the same attractive advantages as those offered by the manufacturer. However, unit-of-use repackagers should conform to all requirements as presented in *Good Repackaging Practices* 〈 1178 〉. There are a number of reasons why repackagers produce unit-of-use packaging: for example, (1) requests from institutions, (2) better inventory control, (3) reduced dispensing times, and (4) variations in some drug therapies.

The packaging of a unit-of-use system may be a multiple container or a single-unit container. A unit-of-use system may contain a drug product in a liquid, semisolid, or solid dosage form (see also *FDA Guidance for Industry on Container Closure Systems for Packaging Human Drugs and Biologics*). [NOTE—The terms "unit-of-use package" and "unit-of-use container" may be used interchangeably.]

The Poison Prevention Packaging Act (PPPA) of 1970 requires in certain cases the use of special packaging—child-resistant and senior-friendly. Child-resistant packaging protects children from serious injury or illness resulting from ingesting or handling hazardous products including drugs.

Because drugs packaged in unit-of-use packaging are intended to be dispensed to the consumer without repackaging by the pharmacist, the manufacturer or repackager is responsible for the special packaging of PPPA-regulated substances in unit-of-use containers (l6 CFR 1701.1).

TYPES OF CONTAINERS FOR UNIT-OF-USE

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Unit-of-use containers are required to be child-resistant if they are intended to be dispensed directly to the patient pursuant to a prescription. Unit-of-use packaging intended for institutional or hospital use may or may not be required to be child-resistant. Unit-of-use containers that are child-resistant single-unit containers include supported blisters, such as separate, peel, push, and tear notch, and enclosed or in-card blisters, such as pull tabs and slide packs. Blister packaging is discussed in the general chapter *Packaging Practice—Repackaging a Single Solid Oral Drug Product into a Unit-Dose Container* ⟨ 1146 ⟩. Unit-of-use containers that are multiple-unit containers include glass and plastic containers.

Single-Unit Container

A single-unit container is one that is designed to hold a quantity of drug product intended for administration as a single dose or a single finished device intended for use promptly after the container is opened. Preferably, the immediate container and/or the outer container or protective packaging shall be so designed as to show any evidence of tampering with the contents. Each single-unit container shall be labeled to indicate the identity, quantity, and/or strength, name of the manufacturer, lot number, and expiration date of the article.

Unit-Dose Container

A unit-dose container is a single-unit container for articles intended for administration by other than the parenteral route as a single dose, directly from the container.

Single-Dose Container

A single-dose container is a single-unit container for articles intended for parenteral administration only. It is labeled as such.

Multiple-Unit Container

A multiple-unit container is a container that permits withdrawal of successive portions of the contents without changing the strength, quality, or purity of the remaining portion.

PACKAGING FABRICATION MATERIALS

Packaging fabrication materials include substances used to manufacture packaging containers such as glass, plastics (including high-density polyethylene (HDPE), low-density polyethylene (LDPE), polyethylene terephthalate, polyethylene terephthalate G and polypropylene (PP), other resins, and other materials as listed in the general test chapter *Containers* ⟨ 661 ⟩ and in the *FDA Guidance for Industry on Container Closure Systems for Packaging Human Drugs and Biologics*.

Glass

Any glass packaging material used in the immediate container should meet the glass test requirements for *Limits for Glass Types* and *Chemical Resistance—Glass Containers: Powdered Glass Test, Water Attack at 121°*, and *Arsenic* under general test chapter *Containers* ⟨ 661 ⟩.

Plastic

Any plastic packaging material used in the immediate container should meet the plastic test requirements for *Plastics* in the general test chapters *Containers* ⟨ 661 ⟩ and *Containers—Permeation* ⟨ 671 ⟩. Depending on the type of plastic packaging material used, the packaging material meets the requirements for *Biological Tests—Plastics and Other Polymers, Physicochemical Tests—Plastics, Polyethylene Containers, Polyethylene Terephthalate Bottles and Polyethylene Terephthalate G Bottles,* and *Polypropylene Containers* under general test chapter *Containers* ⟨ 661 ⟩.

The test for moisture vapor transmission may be carried out as described in the general test chapter *Containers—Permeation* ⟨ 671 ⟩ for multiple-unit and unit-dose containers.

PACKAGING CLOSURE TYPES

Reclosables and nonreclosables may be used for solid, semisolid, and liquid dosage forms. Both must be packaged in compliance with the 16 CFR 1700.15 standards.

Reclosables

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Reclosables are containers with suitable closures that may incorporate tamper evidence and child-resistance capabilities. Reclosables may be used for glass or plastic containers.

Nonreclosables

Nonreclosables are containers with closures that are nonreclosable, such as blisters, sachets, strips, and other single-unit containers. Nonreclosables may include packs such as cold-formed foil blisters, foil strip packs, and PVC/Aclar combining multilayer materials that are thermo-formed or cold-formed foil blisters (see *Packaging Practice—Repackaging a Single Solid Oral Drug Product into a Unit-Dose Container* ⟨ 1146 ⟩). Nonreclosables may be child resistant depending on the intended use and place of use. Household nonreclosables are subject to the PPPA as defined in 16 CFR 1700.14. However, because of some unit-dose designs, not all unit-dose packages comply with the PPPA.

LABELING

The unit-of-use containers are labeled to include expiration dates, the manufacturer's lot number, the NDC designation, and bar codes as provided in the *Labeling* section of the *General Notices and Requirements* under *Preservation, Packaging, Storage, and Labeling* and in *Good Repackaging Practices* ⟨ 1178 ⟩. Some of the advantages of having bar codes on the label include reduced medication errors, improved inventory control, and improved access to medication identity. The labeling covers information placed in the container by the manufacturer (see *General Notices and Requirements*). Acceptable labeling can range from the full labeling as for multiple-unit containers to an abbreviated labeling when the container is too small to include all the text. Full labeling may also be provided on the carton if it is not present on the immediate container.

REPACKAGING AND REPROCESSING

Unit-of-use containers are reprocessed or repackaged as instructed by the manufacturer or as directed in the general test chapter *Containers* ⟨ 661 ⟩ or in the general information chapter *Packaging Practice—Repackaging a Single Solid Oral Drug Product into a Unit-Dose Container* ⟨ 1146 ⟩. A unit-of-use package that is a blister package may not be reprocessed by a pharmacist once it has been deblistered from a unit-dose container (see *General Notices and Requirements* for application of the appropriate beyond-use date for a multiple-unit or unit-dose container). Deblistering is the process of removing medication from a blister-type container. However, under current Good Manufacturing Practices (cGMPs) and tight quality controls, the manufacturer or contract repackager may repackage and reprocess unit-of-use containers.

INFORMATION FROM MANUFACTURERS

The manufacturer should provide appropriate stability information that can be used to determine appropriate labeling, storage, and shipping statements that will properly inform patients and practitioners. The manufacturer may make other assurances based on product information on packaging and distribution arrangements. In the event that a product is not to be repackaged, the manufacturer may so state in the labeling. The manufacturer also includes labeling and information suitable for optimal handling by the practitioner and the patient. The labeling and information should be bar coded to eliminate medication error and promote medication traceability.

RESPONSIBILITY OF THE DISPENSER

Labeling

The labeling on a unit-of-use container also includes a label added at the dispensing stage by the pharmacist. Prior to dispensing the unit-of-use package, the dispenser shall add label(s) that provide the following information:

1. the name of the patient;
2. the name and strength, the directions for use as prescribed by a doctor or health-care provider, and the name of the prescriber; and
3. any storage instruction, beyond-use date, and other information as deemed appropriate by federal and state laws.

In the pharmacy setting, pharmacists are encouraged to use bar codes, in conjunction with computerized prescription orders, to confirm that the right drug is being dispensed to the right patient. Bar coding would minimize errors and create opportunity for medication traceability and accountability.

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Information to Patient

Patients must be given information that applies to the specific prescription being dispensed.

QUALITY CONTROL OF PACKAGING SYSTEM

The packaging system shall meet the general considerations for system suitability, protection, safety, and performance characteristics as described in *FDA Guidance for Industry on Container Closure Systems for Packaging Human Drugs and Biologics*, in the general test chapters *Containers* ⟨661⟩ and *Containers—Permeation* ⟨671⟩, and in the general information chapter *Packaging Practice—Repackaging a Single Solid Oral Drug Product into a Unit-Dose Container* ⟨1146⟩.

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Expert Committee : (PS05) Packaging and Storage 05
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Sustained response and long-term safety of eculizumab in paroxysmal nocturnal hemoglobinuria

Anita Hill, Peter Hillmen, Stephen J. Richards, Dupe Elebute, Judith C. Marsh, Jason Chan, Christopher F. Mojcik, and Russell P. Rother

Paroxysmal nocturnal hemoglobinuria (PNH) is a hematologic disorder characterized by clonal expansion of red blood cells (RBCs) lacking the ability to inhibit complement-mediated hemolysis. Eculizumab, a humanized monoclonal antibody that binds the C5 complement protein, blocks serum hemolytic activity. This study evaluated the long-term safety and efficacy of eculizumab in 11 patients with PNH during an open-label extension trial. After completion of an initial 12-week study, all patients chose to participate in the 52-week extension study. Eculizumab, administered at 900 mg every 12 to 14 days, was sufficient to completely and consistently block complement activity in all patients. A dramatic reduction in hemolysis was maintained throughout the study, with a decrease in lactate dehydrogenase (LDH) levels from 3110.7 IU/L before treatment to 622.4 IU/L ($P = .002$). The proportion of PNH type III RBCs increased from 36.7% at baseline to 58.4% ($P = .005$). The paroxysm rate of days with gross evidence of hemoglobinuria per patient each month decreased from 3.0 during screening to 0.2 ($P < .001$) during treatment. The median transfusion rate decreased from 1.8 U per patient each month before eculizumab treatment to 0.3 U per patient each month ($P = .001$) during treatment. Statistically significant improvements in quality-of-life measures were also maintained during the extension study. Eculizumab continued to be safe and well tolerated, and all patients completed the study. The close relationship between sustained terminal complement inhibition, hemolysis, and symptoms was demonstrated. (Blood. 2005; 106:2559-2565)

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Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a hematopoietic stem-cell disorder characterized by red blood cell (RBC) destruction, anemia, hemoglobinuria, and thrombosis. The intravascular hemolysis in PNH is continuous, with episodes of dark urine, or paroxysms, occurring at times of particularly brisk hemolysis. Ongoing hemolysis and/or insufficient hematopoiesis often result in transfusion dependence. Hemolysis in patients with PNH can be monitored by levels of the enzyme lactate dehydrogenase (LDH), which is typically elevated and can exceed 20 times the upper limit of normal during severe paroxysms.[1-3] There is no effective treatment for the ongoing hemolysis in PNH.

PNH results from the clonal expansion of somatically mutated hematopoietic stem cells. The predominant mutation results in a functional deficiency in phosphatidylinositol glycan class A (PIG-A), a protein that is critical for the biosynthesis of the glycosylphosphatidylinositol (GPI) anchor, a mechanism by which various proteins are attached to the cell membrane.[4,5] Consequently, there is a partial (type II) or complete (type III) deficiency of GPI-anchored proteins on the surfaces of PNH hematopoietic stem cells and their progeny. Two such proteins are the complement inhibitors CD55 and CD59. CD55 inhibits complement at the level of C3, whereas CD59 prevents terminal complement components from forming the hemolytic membrane pore (C5b-9).[6-8] Deficiency of these complement inhibitors renders PNH RBCs sensitive to complement-mediated lysis.[7]

Eculizumab is a humanized monoclonal antibody that specifically targets the complement protein C5 and prevents its cleavage.[9] C5 is the point at which the pathways of complement activation converge, and it is the first protein of terminal complement assembly. Complement inhibition at this stage blocks the generation of C5a and the formation of C5b-9 while it preserves early complement components that are critical for the clearance of microorganisms and immune complexes.[10]

We previously reported the outcome of an open-label study of eculizumab in patients with PNH.[2] Results of this 12-week study demonstrated a dramatic reduction in hemolysis and a concomitant increase in the proportion of PNH type III RBCs. In addition, this initial study showed a marked decrease in the rates of paroxysms and blood transfusions and an improvement in quality of life. Here we report the results of a 1-year follow-up study designed to assess the long-term efficacy and safety of eculizumab in patients with PNH.

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Several of the authors (J.C., C.F.M., R.P.R.) are employed by Alexion, whose potential product was studied in the present work. P.H. reports serving as a consultant to Alexion, and A.H. and P.H. have each received an educational grant from the company.

An Inside *Blood* analysis of this article appears at the front of this issue.

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Patients, materials, and methods

Trial design

The acute-phase study was an initial 12-week, open-label trial of eculizumab in 11 patients with PNH and has been described previously in detail.[2] The current study was an open-label extension of that acute-phase study. All 11 patients from the acute-phase study enrolled in the extension study. Patients were allowed concomitant therapy—with the exception of whole blood, which contains C5—at the discretion of their treating physicians. Two of 11 patients had a history of thrombosis before eculizumab treatment, and 6 of 11 patients were on warfarin therapy before and during the trial.

The trial was approved by the Leeds (West) Research Ethics Committee, United Kingdom, and was performed according to the International Conference on Harmonisation and Good Clinical Practice Standards. All patients gave written informed consent and were treated with eculizumab.

Eculizumab administration

All patients entered the extension study on a maintenance dose of eculizumab (at the conclusion of the acute-phase study). This maintenance dose of 900 mg intravenously every 14 days was continued throughout the extension study period. Two patients, however, required that the dosing interval be shortened to every 12 days so that consistent and complete complement inhibition could be maintained.

Clinical investigations

As in the acute-phase study, data were obtained in the open-label extension study on the pharmacokinetics (PK), pharmacodynamics (PD), and immunogenicity of eculizumab, indicators of hemolysis, PNH clone size, paroxysm and transfusion rates, and quality-of-life measurements. The trigger for transfusion before and during the study remained unchanged for each patient and was based on a combination of hemoglobin levels and the occurrence of symptoms resulting from anemia, hemolysis, or both. This information has been described in detail elsewhere.[2]

Statistical analysis

Biochemical values were compared using the paired Student t test, change of transfusion and paroxysm rates were analyzed using the Wilcoxon signed rank test, quality-of-life measurements were analyzed using mixed-effect analysis of variance, and comparison of the number of days with paroxysms was analyzed using the Fisher exact test.

Results

Pharmacokinetics and pharmacodynamics

In the acute-phase study, 10 of 11 patients maintained sufficient levels of eculizumab (≥ 35 μg/mL) for terminal complement to be sufficiently inhibited (≤ 20% serum hemolytic activity) for the duration of the 12-week treatment period (Figure 1, weeks 0-12). During the extension study, 9 of 11 patients continued to show complete complement blockade throughout the 52-week treatment period (Figure 1, weeks 12-64). The 2 patients whose serum eculizumab levels decreased below 35 μg/mL (Figure 1A, lines 1-2) showed a return of serum hemolytic activity (Figure 1B, lines

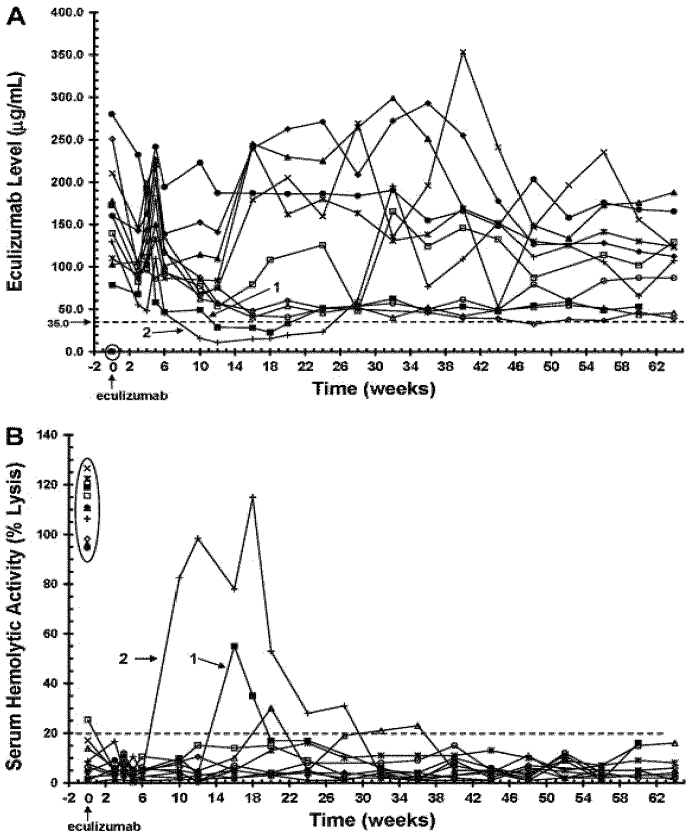


Figure 1. PK and PD analyses of eculizumab in patients with PNH. Initiation of eculizumab dosing is indicated at time 0 on the x axis. (A) Serum levels of eculizumab in 11 patients with PNH during the 64 weeks of treatment. The dashed line indicates the level of eculizumab required to completely block complement activity (≥ 35 μg/mL). Time 0 shows levels of eculizumab before (encircled) and 1 hour after dosing, whereas all other time points represent trough values. Two patients with trough levels of eculizumab below 35 μg/mL during the maintenance dosing are identified (patients 1 and 2). (B) Serum hemolytic activity (PD) during the 64-week treatment period, as determined by the ability of serum to lyse antibody-presensitized chicken erythrocytes. The dashed line indicates the percentage of hemolytic activity at which complement is considered effectively inhibited (≤ 20%). Time 0 shows serum hemolytic activity before (encircled) and 1 hour after dosing, whereas all other time points represent trough values. Two patients with trough serum hemolytic activity values above 20% are identified (patients 1 and 2).

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1-2). However, a reduction in the dosing interval from 900 mg every 14 days to 900 mg every 12 days (initiated between weeks 18 and 24) in the 2 breakthrough patients was adequate to keep trough levels of eculizumab higher than 35 μg/mL (Figure 1A), thereby completely blocking serum complement activity for the remainder of the extension study (Figure 1B).

Measures of hemolysis

Lactate dehydrogenase (LDH) is a standard biochemical measure of intravascular hemolysis, and levels are frequently elevated in patients with PNH.[1-3] The immediate reduction in LDH levels observed in all patients during the acute-phase study was maintained during the extension study (Figure 2). Two patients demonstrated transient increases in LDH levels (Figure 2, lines 1-2) that correlated temporally with the breakthroughs in serum complement activity shown in Figure 1. As complement blockade was restored, LDH levels returned to near-normal values.

Levels of various markers of hemolysis and platelet counts during eculizumab treatment are shown in Table 1. LDH levels decreased from a mean of 3110.7 ± 598.4 IU/L during the 52 weeks before treatment to 594.0 ± 31.7 IU/L and 622.4 ± 41.1 IU/L (normal range at Leeds Teaching Hospitals, 150-480 IU/L) during the 12 and 64 weeks of treatment, respectively ($P = .002$ for 64-week comparison in all patients). Similarly, aspartate aminotransferase (AST) levels, another marker of hemolysis, decreased from a mean baseline value of 76.2 ± 16.0 IU/L to 26.2 ± 2.3 IU/L and 30.1 ± 3.2 IU/L (normal range at Leeds Teaching Hospitals, 10-40 IU/L) during the 12 and 64 weeks of treatment, respectively ($P = .02$ for 64-week comparison in all patients). The dramatic reduction in hemolysis during eculizumab treatment was demonstrated in both noncytopenic (platelet count $\geq 150 \times 10^9$/L) and cytopenic (platelet count $< 150 \times 10^9$/L) patient populations. Levels of haptoglobin, hemoglobin, and bilirubin and numbers of reticulocytes and platelets did not change significantly in the comparison of prestudy values and 64-week treatment values.

Proportions of PNH blood cells

Clonal expansion of hematopoietic stem cells with a reduction in or an absence of GPI-linked membrane proteins (type II or type III cells, respectively) is a hallmark of PNH. The effect of eculizumab on the proportions of various PNH blood-cell types was assessed (Table 2). The proportion of PNH type III RBCs relative to the total RBC population increased from 36.7% ± 5.9% at baseline to 59.2% ± 8.0% and 58.4% ± 8.5% during the 12 and 64 weeks of treatment, respectively ($P = .005$ for 64-week comparison in all patients). The increase in the proportion of PNH type III RBCs was more pronounced in patients without cytopenia than in those with cytopenia. However, the lower proportion of type III RBCs in patients with cytopenia was a direct result of the dilutional effect from the increased administration of normal RBCs because of the higher transfusion requirement in this patient group. The proportion of PNH type II RBCs increased from 5.3% ± 1.4% before treatment to 13.2% ± 2.4% ($P = .013$) during the 64 weeks of treatment. Mean proportions of type III neutrophils and platelets were greater than 90% before eculizumab therapy and were stable during the study.

Paroxysm and transfusion rates

The presence of hemoglobin in the urine (hemoglobinuria) is characteristic of PNH and a central component of periodic exacerbations known as paroxysms. In the eculizumab study, paroxysm rates were defined as the number of days with the presence of gross hemoglobinuria, defined as a colorimetric urine score of 6 or greater on a scale of 1 to 10.[2] In patients whose urine scores were assessed, the paroxysm rate decreased from 3.0 paroxysms per patient each month before eculizumab treatment to 0.1 paroxysms per patient each month during the initial 12 weeks and 0.2 paroxysms per patient each month during the total 64 weeks of treatment (Figure 3; $P < .001$).

During the extension study, 2 patients did not sustain levels of eculizumab necessary to consistently block complement (Figure 1). 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. A detailed analysis of the dosing interval between visits 4 and 5 in one of the breakthrough patients showed that sufficient levels of eculizumab (PK ≥ 35 μg/mL) were present to completely block serum hemolytic activity (PD ≤ 20% hemolytic activity) for the first 12 days (Figure 4). Effective complement blockade during these 12 days was reflected by normal urine color scores and low LDH and AST levels. On the 13th and 14th days of the dosing interval, a paroxysm occurred, as evidenced by severe hemoglobinuria (black urine), dysphagia, and dramatic increases in LDH and AST levels. These events correlated with insufficient levels of eculizumab (PK) and the return of serum hemolytic activity (PD). The patient was again dosed on day 14, which resulted in the resolution of hemoglobinuria and dysphagia by the next morning. A reduction in the dosing interval from 14 to 12 days between visits 5 and 9 was sufficient to maintain the levels of

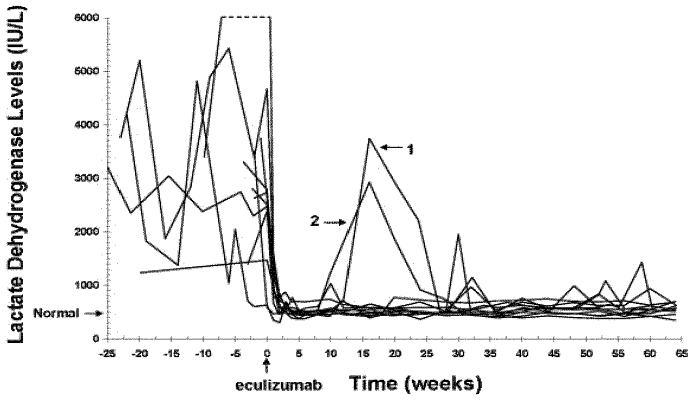


Figure 2. LDH levels in patients with PNH before and during eculizumab treatment. Initiation of eculizumab dosing is indicated at time 0 on the x axis. LDH values are shown for 11 patients with PNH for 25 weeks before and 64 weeks during eculizumab treatment. (Normal) Upper limit of normal of the LDH range at the Leeds Teaching Hospital. The dashed line indicates off-scale points from one patient with a peak value of 12 100 IU/L. Two patients who experienced a return of serum hemolytic activity during treatment are identified (patients 1 and 2).

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Table 1. Changes in levels of various markers of hemolysis and platelet counts during eculizumab therapy

Biochemical marker	Normal range	Time of analysis‖			P*
		Before study†	12 wk	64 wk	
LDH, IU/L					
All patients	150-480	3110.7 ± 598.4	594.0 ± 31.7	622.4 ± 41.1	.002
Patients without cytopenia‡	NA	3965.0 ± 971.7	617.2 ± 24.3	657.1 ± 54.8	—
Patients with cytopenia§	NA	2085.0 ± 267.0	566.3 ± 65.5	580.8 ± 63.2	—
AST, IU/L					
All patients	10-40	76.2 ± 16.0	26.2 ± 2.3	30.1 ± 3.2	.02
Patients without cytopenia	NA	88.5 ± 28.3	24.4 ± 3.8	30.8 ± 5.7	—
Patients with cytopenia	NA	61.4 ± 9.8	28.4 ± 2.4	29.1 ± 2.3	—
Haptoglobin, g/L					
All patients	0.5-2	0.06 ± 0	0.07 ± .01	0.14 ± 0.07	NS
Patients without cytopenia	NA	0.06 ± 0	0.08 ± .02	0.20 ± 0.12	—
Patients with cytopenia	NA	0.06 ± 0	0.06 ± 0	0.08 ± 0.01	—
Hemoglobin, g/dL					
All patients	11.5-16	10.0 ± 0.4	10.3 ± 0.4	10.4 ± 0.4	NS
Patients without cytopenia	NA	10.4 ± 0.5	10.9 ± 0.6	10.8 ± 0.5	—
Patients with cytopenia	NA	9.5 ± 0.7	9.6 ± 0.4	9.9 ± 0.5	—
Bilirubin, μM					
All patients	3-15	25.9 ± 4.3	28.2 ± 4.4	28.7 ± 4.0	NS
Patients without cytopenia	NA	30.6 ± 7.4	34.9 ± 6.6	34.6 ± 5.9	—
Patients with cytopenia	NA	20.2 ± 2.2	20.2 ± 3.6	21.7 ± 3.6	—
Reticulocytes, $\times 10^{-3}/mm^3$					
All patients	20-80	161.4 ± 25.9	191.2 ± 23.6	189.6 ± 21.8	NS
Patients without cytopenia	NA	200.2 ± 38.4	243.4 ± 26.2	233.2 ± 27.4	—
Patients with cytopenia	NA	114.8 ± 21.9	128.5 ± 15.6	137.3 ± 15.2	—
Platelets, $\times 10^9/L$					
All patients	150-400	183.0 ± 35.3	183.6 ± 37.8	180.8 ± 35.8	NS
Patients without cytopenia	NA	250.2 ± 49.8	256.3 ± 51.8	251.5 ± 47.2	—
Patients with cytopenia	NA	102.2 ± 11.8	96.3 ± 18.7	95.9 ± 19.7	—

— indicates not determined; NA, not applicable; and NS, not significant.
*Comparisons of mean change from the prestudy period to the 64-week treatment period for all patients.
†Mean values during 52-week period before treatment except for AST, which represents the baseline mean.
‡Platelet count ≥ 150×10^9/L; n = 6.
§Platelet count < 150×10^9/L; n = 5.
‖Values are presented as ± standard error (SE).

Table 2. Changes in proportions of PNH blood-cell types during eculizumab treatment

PNH cell type	Proportion of PNH cells, %			P*
	Baseline§	12 wk§	64 wk§	
Type III RBCs				
All patients	36.7 ± 5.9	59.2 ± 8.0	58.4 ± 8.5	.005
Patients without cytopenia†	38.8 ± 3.7	73.5 ± 4.1	67.8 ± 7.0	—
Patients with cytopenia‡	34.2 ± 12.9	42.1 ± 13.8	47.0 ± 16.3	—
Type II RBCs				
All patients	5.3 ± 1.4	7.5 ± 2.1	13.2 ± 2.4	.013
Patients without cytopenia	4.8 ± 1.6	6.8 ± 3.0	12.9 ± 1.9	—
Patients with cytopenia	5.8 ± 2.4	8.4 ± 3.2	13.5 ± 5.0	—
Type III WBCs				
All patients	92.1 ± 4.6	89.9 ± 6.6	91.1 ± 5.8	NS
Patients without cytopenia	95.1 ± 2.3	94.9 ± 2.6	96.1 ± 1.1	—
Patients with cytopenia	88.4 ± 10.2	83.9 ± 14.5	85.2 ± 12.9	—
Type III platelets				
All patients	92.4 ± 2.4	93.3 ± 2.8	92.8 ± 2.6	NS
Patients without cytopenia	93.2 ± 1.4	95.8 ± 2.1	95.0 ± 0.8	—
Patients with cytopenia	91.6 ± 5.4	90.2 ± 5.6	90.2 ± 5.8	—

— indicates not determined; WBCs, white blood cells; and NS, not significant.
*Comparisons of mean change from baseline to the 64-week treatment period for all patients.
†Platelet count ≥ 150×10^9/L; n = 6.
‡Platelet count < less than 150×10^9/L; n = 5.
§Data are presented as ± SE.

eculizumab necessary to effectively and consistently block serum hemolytic activity and, therefore, intravascular hemolysis in these patients (Figure 4, visit 9).

A statistically significant reduction in the rate of packed red blood cell (PBRC) transfusion was observed in the acute-phase study and was maintained during the extension study compared with the rate of transfusion before eculizumab therapy (Table 3). Mean transfusion rates decreased from 2.1 U per patient each month during the 1-year period before treatment to 0.6 U per patient each month during the initial 12-week and to 0.5 U per patient each month during the total 64-week treatment period.

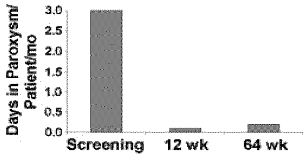
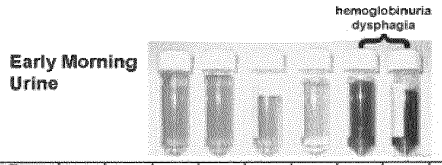


Figure 3. Paroxysm rate in patients with PNH before and during eculizumab treatment. A urine color scale[2] was used to assess the incidence of paroxysms in 8 patients with PNH before and during treatment with eculizumab. Paroxysm was prospectively defined by a urine colorimetric score of 6 or more. Bars represent the paroxysm rates (number of paroxysms per patient per month) during the screening period, during the first 12 weeks, and over the total 64 weeks of eculizumab treatment. Three patients were not included in the analysis either because their pretreatment urine scores were inadvertently not collected (2 patients) or because an iron-chelating agent that resulted in artificially colored urine was administered during the extension study (1 patient).

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Day (visit)	0*(4)	1-8	9	10	11	12	13	0*(5)	1-2	0*(9)
Urine	10	2-3	3	3	3	3	9	10	3	2
LDH	2624	–	784	–	–	697	1687	2917	–	495
AST	119	–	38	–	–	31	87	–	–	–
PK	28	–	51	–	–	35	31	23	–	51
PD	72	–	1	–	–	2	56	56	–	2

Figure 4. Relationship between complement inhibition with eculizumab and various hemolytic parameters and symptoms during a transient breakthrough in serum-complement activity. Urine color, symptoms, biochemical parameters of hemolysis, PK, and PD were assessed during a 14-day eculizumab-dosing interval in a patient with a transient breakthrough in serum hemolytic activity. Eculizumab was administered on day 0 after assay samples were collected. A urine colorimetric score of 6 or greater was considered abnormal (hemoglobinuria). Levels of the hemolytic markers LDH and AST are shown (IU/L). Eculizumab serum concentrations (PK, μg/mL) of ≥ 35 μg/mL were sufficient to maintain a serum hemolytic activity (PD, % serum hemolytic activity) of ≤ 20%, a value known to represent complete complement blockade. The eculizumab-dosing interval was reduced to 12 days between visits 5 and 9. Urine row presents colorimetric scores. — indicates not determined. *Dose of eculizumab.

Similarly, median transfusion rates decreased from 1.8 U per patient each month before treatment to 0.0 U and 0.3 U per patient each month during the first 12 weeks and the total 64 weeks of therapy, respectively ($P = .001$ for 64-week comparison in all patients). The reduction in transfusions was more pronounced in patients without cytopenia; the mean rate in this population decreased from 2.4 U per patient each month during the prestudy period to 0.2 U per patient each month during the 12- and 64-week treatment periods. Transfusion reduction in patients with poor bone marrow reserve (patients with cytopenia) decreased from a mean rate of 1.8 U per patient each month during the 1-year pretreatment period to 1.2 and 0.8 U per patient each month during the 12- and 64-week treatment periods, respectively.

Quality-of-life measurements

Quality-of-life measurements were assessed using the European Organization for Research and Treatment of Cancer (EORTC) QLQ-C30 instrument during the total 64-week treatment period and were compared with baseline values (Table 4). Significant improvements in quality-of-life domains that were demonstrated in the acute-phase study were maintained in the extension study, including global health status ($P = .009$), physical functioning ($P < .001$), emotional functioning ($P < .001$), cognitive functioning ($P = .001$), fatigue ($P < .001$), dyspnea ($P < .001$), and insomnia ($P = .031$). In addition, changes in role functioning ($P = .003$) and pain domains ($P = .023$) achieved significance during the 64-week treatment period ($P < .001$). The constipation domain showed a significant increase over the treatment period. Taken together, these results indicate that various parameters of quality of life in patients with PNH rapidly improve with eculizumab therapy and that these changes are maintained for extended lengths of time.

Table 4. Quality-of-life assessment during eculizumab treatment

Domain*	Mean baseline score†	64-wk change from baseline score‡	P§
Global health status	56.1	13.8	.009
Physical functioning	70.9	14.3	<.001
Emotional functioning	70.5	12.5	<.001
Role functioning	66.7	14.5	.003
Cognitive functioning	77.3	10.3	.001
Fatigue	47.5	−17.8	<.001
Dyspnea	39.4	−16.6	<.001
Insomnia	30.3	−8.2	.031
Pain	21.2	−8.2	.023
Constipation	3.0	4.1	<.001

*Quality of life was assessed using the European Organization for Research and Treatment of Cancer QLQ-C30 instrument.

†Mean values of linearly transformed scores.

‡Values represent least-square means. Positive change indicates improvement on Global Health Status and Functional scales, and negative change indicates improvement on Symptom scales.

§From a mixed analysis-of-covariance model with visit as a fixed effect, patient as a random effect, and baseline as a covariate.

Safety

All patients completed the extension study. No deaths occurred and no thromboses developed during the 12-month treatment period. In no patients were antibodies against eculizumab detected. All patients had at least one adverse event (AE), and one patient had a serious adverse event (SAE). No patient withdrew because of an AE. The most common AEs were flulike symptoms (4 patients), sore throat (4 patients), pain (3 patients), nausea (3 patients), bruising (3 patients), cough (3 patients), and upper respiratory infection (3 patients).

One patient experienced an SAE during the extension study characterized by neutropenia with extravascular hemolysis, which was thought to have been caused by a viral syndrome. Briefly, the patient had a 5-year history of PNH with aplastic anemia (with a normal neutrophil count) and had been on eculizumab therapy for 14 months. A viral syndrome developed, and during the next several days hemoglobin levels dropped from 10.2 to 5.3 g/dL, whereas serum LDH levels increased only slightly. The patient

Table 3. Changes in transfusion requirements during eculizumab treatment

	Transfusion rates						
	Before study*		12 wk		64 wk		
Patient group	Mean	Median	Mean	Median	Mean	Median	P†
All patients	2.1	1.8	0.6	0.0	0.5	0.3	.001
Patients without cytopenia‡	2.4	2.0	0.2	0.0	0.2	0.1	–
Patients with cytopenia§	1.8	1.8	1.2	0.7	0.8	0.7	–

Transfusion rates are units per patient per month.

– indicates not determined.

*Values during 52-week period before treatment.

†Comparisons of median change from before study to the 64-week treatment period.

‡Platelet count ≥ 150×10^9/L; n = 6.

§Platelet count < 150×10^9/L; n = 5.

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received a transfusion of 4 U PBRCs; at the next study visit, the hemoglobin level had returned to 10.7 g/dL and the platelet count was normal, but the neutrophil count was abnormally low (260/μL). Two weeks later, the neutrophil count had recovered to almost-normal levels (1600/μL). The investigator did not think the SAE was caused by the study medication. The patient remained transfusion independent for the remainder of the extension study.

Discussion

We reported previously that eculizumab therapy in patients with PNH resulted in a dramatic reduction in hemolysis and transfusion requirements. The extension study was conducted to obtain long-term data regarding eculizumab use in this patient population. Here we report the sustained efficacy and safety of eculizumab in a 1-year, open-label extension study.

Serum hemolytic activity in 9 of 11 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. One patient had a breakthrough of serum hemolytic activity during the acute-phase study, and another patient broke through early in the extension study. PK analysis of both breakthrough patients demonstrated a simultaneous decrease in eculizumab level below that required to completely block complement activity (≥ 35 μg/mL).[9] Adjustment of the eculizumab dosing interval in these 2 patients from every 14 days to every 12 days successfully sustained trough levels of eculizumab higher than 35 μg/mL and consistently blocked serum hemolytic activity for the remainder of the extension study. The effective and consistent blockade of complement achieved with the 12-day dosing interval was supported by the resolution of symptoms, including hemoglobinuria and dysphagia, and lower levels of LDH and AST (Figure 4). Taken together, these data illustrate the tight relationship between complement blockade, hemolysis, and symptoms in PNH.

The dramatic reduction in LDH levels that was demonstrated immediately on the administration of eculizumab in the initial study was sustained during the extension study, providing strong evidence that eculizumab effectively and durably inhibits intravascular hemolysis in patients with PNH. LDH levels were reduced from a mean of 3110.7 IU/L during the 52-week period before treatment to a mean of 622.4 IU/L during the 64 weeks of eculizumab therapy. In addition, detailed analysis of PK and PD demonstrated a strong correlation between the return of complement activity, hemolysis, and an increase in LDH levels (Figure 4). These data confirm that LDH levels can be used as an accurate measure of intravascular hemolysis in PNH, and they provide evidence that effective complement blockade during eculizumab therapy can be determined by monitoring levels of this enzyme.

LDH levels remained slightly elevated in most patients during eculizumab treatment, suggesting low levels of ongoing hemolysis. Undetectable haptoglobin levels and elevated bilirubin levels also support residual hemolysis in the midst of terminal complement inhibition. This may be attributed to yet undefined, noncomplement-mediated mechanisms of PNH RBC clearance, as recently described.[11] Alternatively, it is possible that a fraction of PNH RBCs is cleared through complement-mediated events before C5, such as a C3b coating and clearance. In this regard, slightly elevated levels of LDH have also been reported in a patient with coexistent PNH and C9 deficiency.[12]

The marked reduction in transfusions demonstrated in the initial eculizumab study was sustained in the extension study. Further, patients who had good bone marrow reserve (those without cytopenia) showed greater reductions in transfusion requirements than did patients who had hypoplasia (those with cytopenia). Importantly, 3 patients with hypoplasia who received transfusions during the acute-phase study were subsequently treated with erythropoietin during the extension study. Two of the 3 patients responded with an increase in reticulocyte counts and a further reduction in transfusion requirements (data not shown). These data suggest that erythropoietin therapy in PNH patients with hypoplasia increases erythropoiesis and that concomitant eculizumab therapy protects against increased hemolysis, with a resultant improvement in response. Three of 11 PNH patients remain transfusion independent after 64 weeks of eculizumab therapy.

Interestingly, eculizumab therapy resulted in dramatic reductions in transfusion requirements even though hemoglobin levels and reticulocyte counts frequently remained fairly constant. However, before eculizumab treatment, hemoglobin levels in patients were artificially maintained through regular transfusions of packed RBCs. Thus, stabilization of hemoglobin levels with a concomitant reduction in or cessation of transfusions likely represents a net increase in hemoglobin levels. These data suggest that the resolution of hemolysis in patients with PNH results in a new steady state hemoglobin level, determined by a combination of the extent of the underlying bone marrow dysfunction, the size of the type III RBC clone, and the new level (if any) of transfusion requirement.

There was a sustained improvement in quality of life for transfusion-dependent PNH patients administered eculizumab, as measured by the EORTC QLQ-C30. Quality-of-life parameters that showed significant improvement include global health status, physical functioning, emotional functioning, role functioning, cognitive functioning, fatigue, dyspnea, insomnia, and pain. Improvements in quality-of-life parameters in PNH patients taking eculizumab will be further investigated in subsequent placebo-controlled trials.

Clinical assessment of PNH symptoms not captured by the QLQ-C30 instrument, such as dysphagia, abdominal pain, and erectile failure, showed complete resolution, or at least dramatic improvement, during eculizumab treatment.[13] These symptoms have been shown to correlate with a large PNH type III clone size, suggesting that they are related to excessive hemolysis.[14] In addition, these symptoms have been attributed to smooth muscle dystonia caused by the scavenging of nitric oxide by free plasma hemoglobin.[14-18] The capacity of free plasma hemoglobin to scavenge nitric oxide during intravascular hemolysis has been demonstrated in patients with sickle cell anemia.[19] The relationship between levels of free plasma hemoglobin and nitric oxide in patients with PNH remains to be elucidated.

Thrombosis accounts for most deaths in patients with PNH. Studies have reported a strong correlation between a large PNH type III neutrophil clone and the occurrence of thrombosis.[14,20] Hall et al[20] reported that in approximately 44% of patients with large PNH clones, venous thrombosis developed in the first 10 years after diagnosis. The tendency toward thrombosis in patients with PNH is thought to be multifactorial in etiology, involving both the absence of GPI-anchored complement inhibitors on the surfaces of circulating platelets and the high levels of intravascular free plasma hemoglobin with the consequent scavenging of nitric oxide.[18,21-25] It is reasonable to hypothesize that thrombosis resulting from either or both of these mechanisms should be reduced by terminal complement blockade. Although no thrombotic events occurred

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during eculizumab treatment, 6 of the 11 patients were on warfarin therapy before and during the study. Further analyses and studies are required to investigate whether eculizumab reduces the risk for thrombosis in patients with PNH.

Eculizumab continued to be safe and well tolerated in the PNH extension study. No deaths occurred and no thromboses developed in this study. Each patient had at least one AE; flulike symptoms and sore throat were the most common. Evaluation of AEs in other placebo-controlled eculizumab studies suggests that the reported AEs in the PNH extension study were similar in type and frequency to those reported in other placebo treatment groups. The single SAE, transient neutropenia, was not thought by the principal investigator to be related to study medication; rather, it was thought to be the result of a viral syndrome. All patients chose to continue treatment in a second extension study, and evaluation of eculizumab therapy in a broader population of PNH patients is under way.

Results of this 1-year extension study showed that eculizumab therapy continues to be safe and well tolerated in PNH patients. Additionally, long-term complement inhibition with eculizumab in this patient population has resulted in sustained reductions in hemolysis and blood transfusions and continued improvement in quality of life.

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Sustained response and long-term safety of eculizumab in paroxysmal nocturnal hemoglobinuria

Anita Hill, Peter Hillmen, Stephen J. Richards, Dupe Elebute, Judith C. Marsh, Jason Chan, Christopher F. Mojcik and Russell P. Rother

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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 cluted 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.).

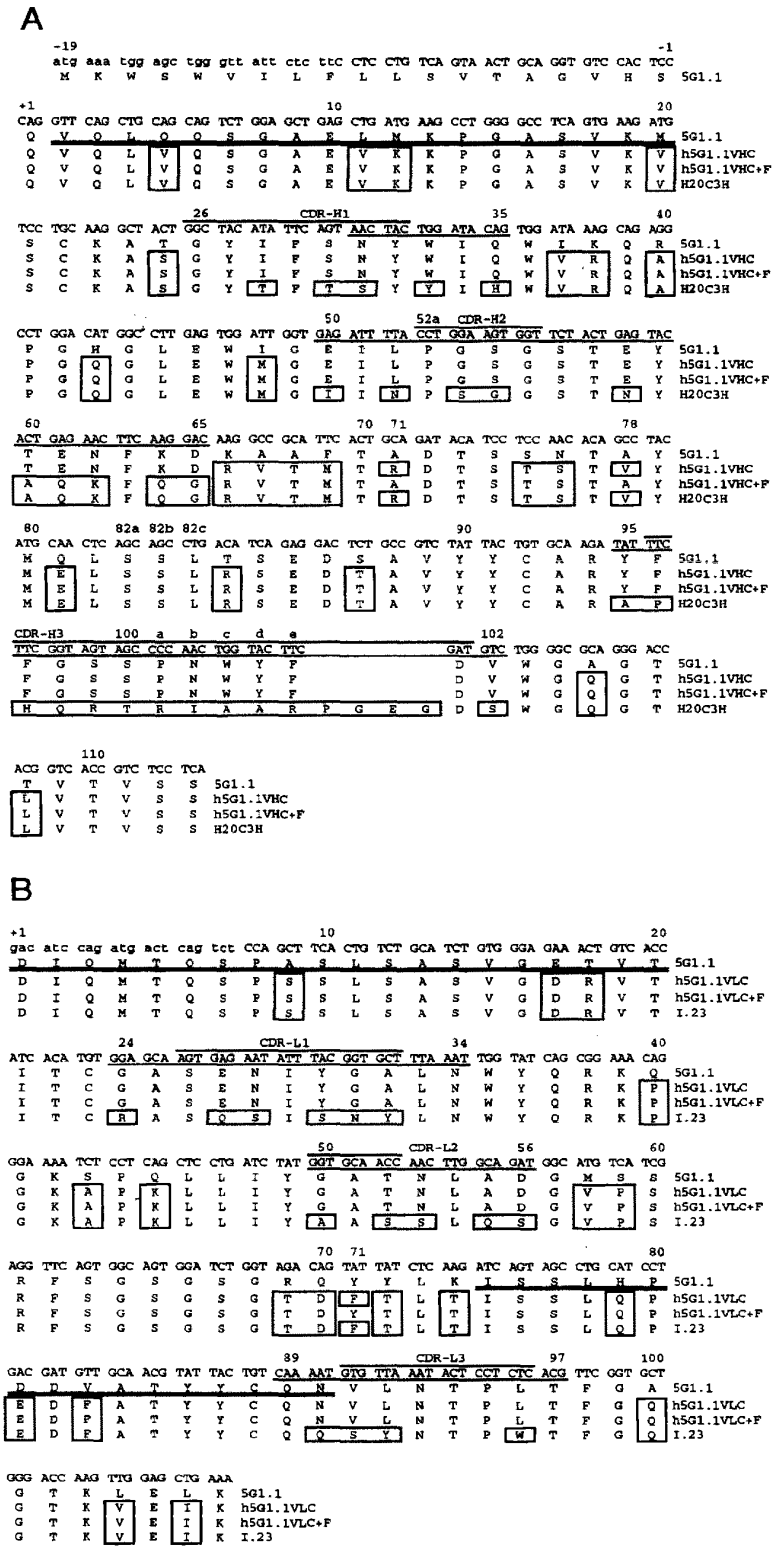
Construction of a humanized scFv

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

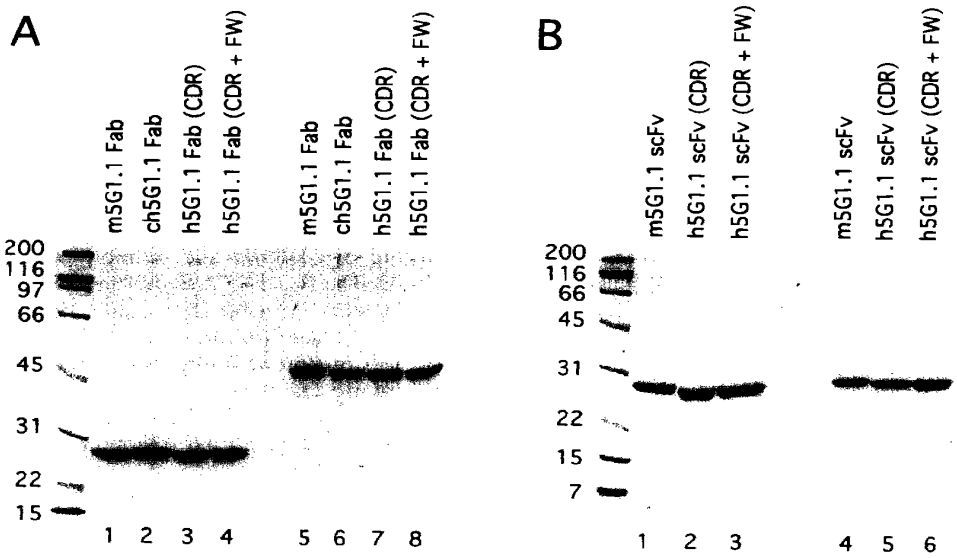


Fig. 2. SDS–PAGE analysis of purified recombinant proteins. (A) Fab fragments from the murine 5G1.1 mAb (m5G1.1 Fab) were produced by papain digestion of the m5G1.1 mAb followed by protein A chromatography to remove undigested antibody and Fc fragments. Recombinant chimeric Fab (ch5G1.1 Fab), CDR-grafted 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.

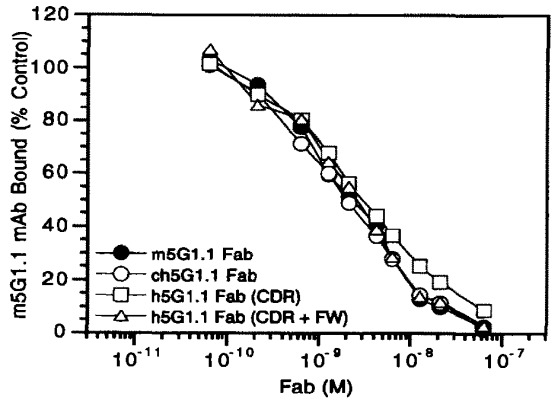


Fig. 3. Inhibition of 5G1.1 mAb binding to human C5 by recombinant Fab. 5G1.1 (0.2 nM) mAb plus the indicated concentrations of recombinant Fab were incubated together, for 2 hr at 37°C in microtiter plates coated with human C5. After washing, bound 5G1.1 mAb was detected using peroxidase-conjugated 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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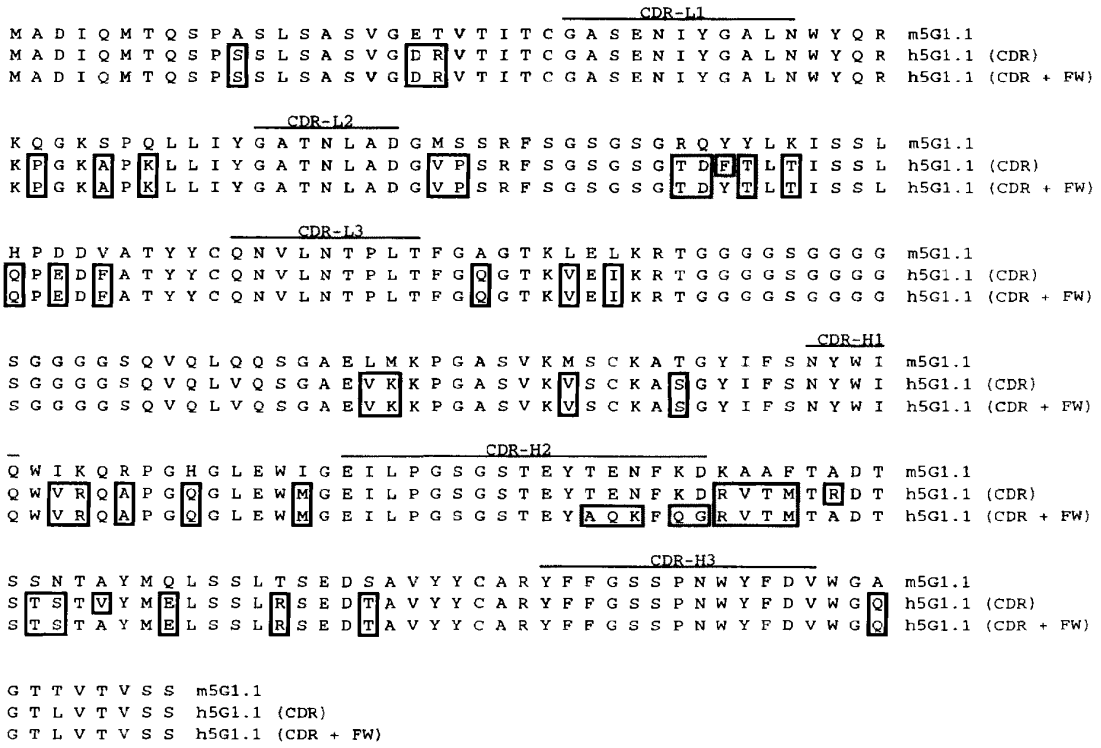


Fig. 4. Sequence of scFv molecules constructed using the 5G1.1 murine variable regions (m5G1.1) or humanized variable regions. The h5G1.1 (CDR) scFv contains the variable regions h5G1.1 VLC and h5G1.1 VHC, while the h5G1.1 scFv (CDR + FW) contains the variable regions h5G1.1 VLC+F and h5G1.1 VHC+F. The 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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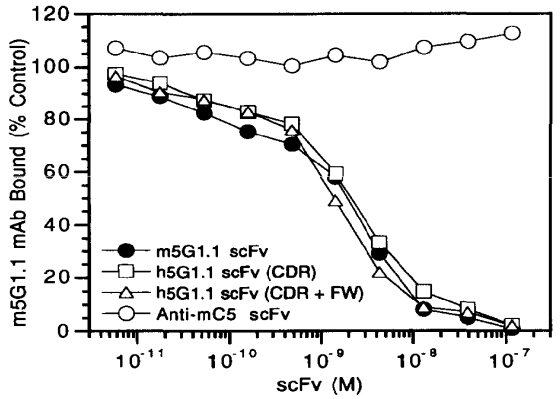


Fig. 5. Inhibition of 5G1.1 mAb binding to human C5 by recombinant scFv. 5G1.1 mAb (0.2 nM) plus the indicated concentrations of recombinant scFv were incubated together for 2 hr at 37°C, in microtiter plates coated directly with human C5. After washing, bound 5G1.1 mAb was detected using peroxidase-conjugated 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.

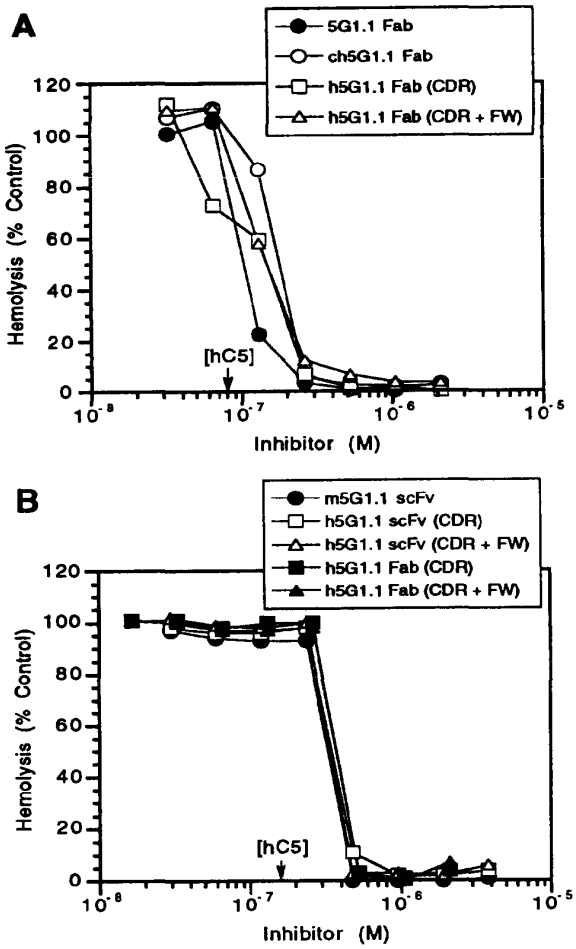


Fig. 6. Inhibition of chicken erythrocyte lysis by recombinant Fab and scFv. Twenty percent (A), or 40% (B), human serum in $GVBS^{++}$ was pre-incubated for 30 min at room temperature, with the indicated concentrations of competitors. Chicken erythrocytes, precoated with rabbit anti-chicken erythrocyte 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

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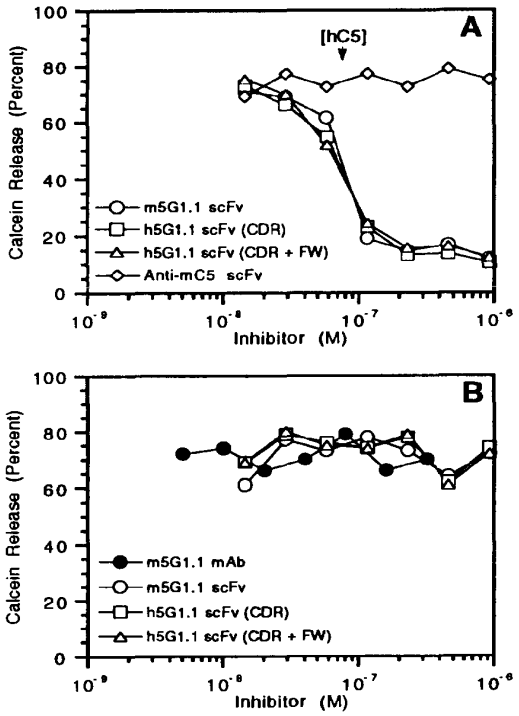


Fig. 7. Protection of porcine aortic endothelial cells (PAEC) from human serum complement by scFv. Calcein-labeled PAEC were incubated with HBSS containing 1% BSA and 0.85 mg/ml purified rabbit anti-PAEC polyclonal antibody for 30 min at 37°C. The cells were washed and incubated with HBSS containing 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.

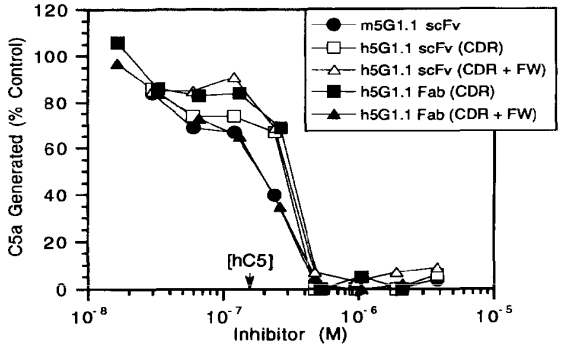


Fig. 8. Inhibition of C5a generation by recombinant Fab and scFv. Supernatants from chicken erythrocyte hemolytic assays performed using 40% human serum, as described in Fig. 5, were assayed for C5a content by ELISA. The C5a concentration obtained in the absence of inhibitor was defined as 100%. Data shown are from a representative experiment of several performed. The predicted hC5 concentration in 40% serum is indicated by the arrow.

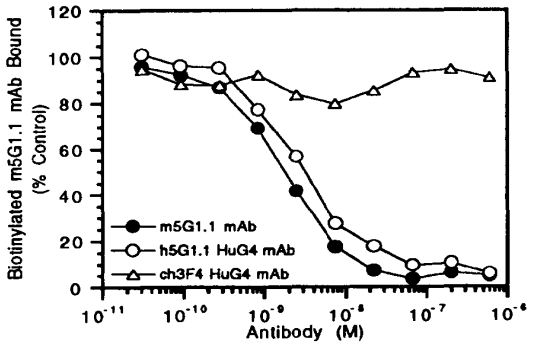


Fig. 9. Inhibition of 5G1.1 mAb binding to human C5 by recombinant humanized h5G1.1 HuG4 mAb. Biotinylated 5G1.1 mAb (0.2 nM), plus the indicated concentrations of unlabeled murine 5G1.1 mAb, recombinant h5G1.1 HuG4 mAb or a negative control recombinant HuG4 mAb (ch3F4 HuG4 mAb), were incubated together 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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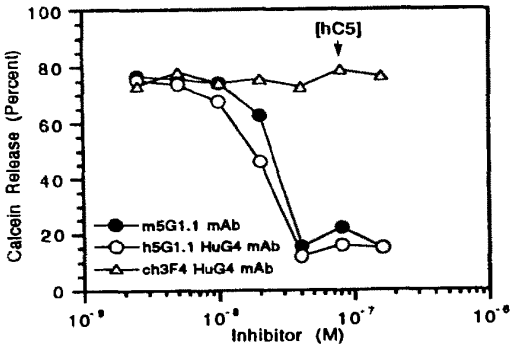


Fig. 10. Protection of porcine aortic endothelial cells from human serum complement by h5G1.1 HuG4 mAb. Calcein-labeled PAEC were incubated with HBSS containing 1% BSA and 0.85 mg/ml purified rabbit anti-PAEC polyclonal antibody for 30 min at 37°C. The cells were washed and incubated with HBSS containing 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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HUMANIZED PORCINE VCAM-SPECIFIC MONOCLONAL ANTIBODIES WITH CHIMERIC IgG2/G4 CONSTANT REGIONS BLOCK HUMAN LEUKOCYTE BINDING TO PORCINE ENDOTHELIAL CELLS

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Abstract—Discordant xenografts surviving the initial hyperacute rejection phase may be subject to cellular rejection processes mediated by infiltrating leukocytes including T cells, NK cells and monocytes. The stable adhesion of these cell types to endothelial cells is due to the molecular interaction of the integrins VLA-4 and LFA-1 with their ligands vascular cell adhesion molecule (VCAM) and ICAM-1 present on the endothelial cells. Human VLA-4 binds to porcine VCAM, and blocking mAbs specific for porcine VCAM have been developed. We have localized the epitope of the anti-porcine VCAM blocking mAbs 2A2 and 3F4 to domains 1 and 2, respectively. Humanized antibodies (IgG4 isotype) were constructed from these anti-porcine VCAM antibodies and demonstrated to inhibit adhesion of Ramos, Jurkat and YT cells, as well as purified resting and activated human T cells, to porcine aortic endothelial cells (PAEC). These cell types express both LFA-1 as well as VLA-4, suggesting blockade of human VLA-4 interaction with porcine VCAM may alone be sufficient to significantly impair adhesion of human leukocytes to porcine endothelial cells. The chimeric anti-porcine VCAM (pVCAM) HuG4 antibodies promoted increased adhesion of Fc receptor (FcR) positive cells such as U937 monocytic cells to PAEC. In contrast, chimeric anti-porcine VCAM antibodies created using the CH1 and hinge region from human IgG2 and the CH2 and CH3 regions from human IgG4 (HuG2/G4 antibodies) inhibited binding of FcR positive cells to PAEC. These chimeric anti-pVCAM antibodies should allow delineation of the *in vivo* role of VLA-4/VCAM interaction in porcine-to-primate xenotransplants. Further, the design of the HuG2/G4 antibodies should render them efficacious in multiple settings requiring elimination of FcR binding. © 1997 Elsevier Science Ltd.

Key words: adhesion molecules, antibodies, Fc receptors, inflammation, xenotransplantation.

INTRODUCTION

Although much progress has been made in elucidating the molecular mechanisms of hyperacute rejection of xenotransplants (reviewed in Somerville and D'Apice, 1993), the mechanisms of cell mediated rejection of xenografts remain unclear. The cellular infiltrate observed 3 days following transplantation of guinea pig hearts into complement (C)-depleted rats is comprised predominantly of macrophages and natural killer cells, with low numbers of T lymphocytes present (Blakely *et al.*, 1994). Similarly, the cellular infiltrate present 6 days after transplantation of porcine fetal islet clusters into rats is comprised predominantly of macrophages with low numbers of T lymphocytes present primarily at the periphery of the graft (Wallgren *et al.*, 1995). *In vitro* porcine aortic endothelial cells (PAEC) as well as antigen presenting peripheral blood cells can directly activate human CD4+ and CD8+ T lymphocytes (Rollins *et al.*, 1994*a*; Murray *et al.*, 1994; Yamada *et al.*, 1995). Thus the initial phases of cellular rejection of xenotransplants are likely to involve binding and activation of multiple types of leukocytes including not only T lymphocytes but also natural killer cells and monocytes.

The stable adhesion of lymphocytes, natural killer cells, and monocytes to endothelial cells is mediated predominantly by the interaction of the integrins LFA-1 (CD11a/CD18) and VLA-4 (CD49d/CD29) with their cognate ligands ICAM-1 and VCAM-1 on endothelial cells (Allavena *et al.*, 1991; Jonjic *et al.*, 1992). For both monocytes (Luscinskas *et al.*, 1994) and T lymphocytes (Alon *et al.*, 1995; Luscinskas *et al.*, 1995) the binding of VLA-4 to VCAM is an essential component of the *in vitro* adhesive interactions required for stable arrest under

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conditions of flow. *In vitro*, the transmigration of NK cells and monocytes across human umbilical vein endothelial cell (HUVEC) monolayers also utilizes both LFA-1/ICAM-1- and VLA-4/VCAM-dependent pathways (Bianchi *et al.*, 1993; Meershaert and Furie, 1994; Chuluyan *et al.*, 1995). *In vivo*, the interaction of LFA-1 with ICAM-1 may not be completely essential for extravasation of lymphocytes or monocytes since both of these cell types are present in inflammatory infiltrates of leukocyte adhesion deficiency patients who have a genetic deficiency in CD18 and thus lack integrins such as LFA-1 (Hawkins *et al.*, 1992). Finally, numerous studies have demonstrated the potential of blocking antibodies directed against LFA-1 (Issekutz, 1992; Scheynius *et al.*, 1993), VLA-4 (Issekutz, 1991; Yednock *et al.*, 1992; Chisholm *et al.*, 1993), ICAM-1 (Zeng *et al.*, 1994), or VCAM (Orosz *et al.*, 1993; Silber *et al.*, 1995) to inhibit the accumulation of lymphocytes at inflammatory sites in animals.

For xenogeneic cellular interactions it is not yet fully defined which adhesion molecule interactions will function across species barriers. It is likely that at least some xenogeneic receptor–ligand interactions will be nonfunctional. For example, mouse LFA-1 does not bind to human ICAM-1 (Johnston *et al.*, 1990). To explore the molecular basis of adhesive interactions between distinct classes of adhesion molecules on human and porcine cells we have cloned porcine E-selectin and VCAM-1 and have demonstrated that both are capable of mediating the adhesion of human leukocytes (Rollins *et al.*, 1994*a*; Mueller *et al.*, 1995). Further, we have developed murine monoclonal antibodies (mAbs) specific for porcine VCAM which block binding of human VLA-4 to porcine VCAM (Mueller *et al.*, 1995). The functional blocking activity of these antibodies suggests that they will be useful in suppressing the cellular response to a porcine xenograft. However, murine mAbs are not ideally suited for use in primates due to their short half-life and high immunogenicity (Khazaeli *et al.*, 1994). Here we have utilized antibody engineering strategies to create humanized anti-porcine VCAM antibodies that block adhesion of human natural killer cells, monocytes, and T lymphocytes to activated PAEC. Furthermore, we show the choice of human isotype has critical implications for the potential therapeutic use of humanized antibodies in xenotransplantation.

MATERIALS AND METHODS

Cells

Ramos, Jurkat and U-937 cells were obtained from the American Type Culture Collection, YT cells were the gift of Dr J. M. Wang (National Cancer Institute, Frederick, MD, U.S.A.), porcine aortic endothelial cells (PAEC) were obtained from Cell Systems (Kirkland, WA, U.S.A.) and 293-EBNA cells were obtained from Invitrogen (San Diego, CA, U.S.A.). U-937 cells were maintained in RPMI 1640 supplemented with 15% heat-inactivated FBS and 2 mM L-glutamine. Ramos and Jurkat cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 20 mM HEPES, 50 μM β-mercaptoethanol, 100 units/ml penicillin and 100 μg/ml streptomycin. YT were maintained in RPMI 1640 containing 10% heat-inactivated FBS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. PAEC were maintained in medium A consisting of M199 supplemented with 20% heat-inactivated FBS, 0.05 mg/ml ECGF (Biomedical Technologies, Stoughton, MA, U.S.A.), 20 mM HEPES and 0.1 mg/ml heparin, and were used within three passages. 293-EBNA cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and 250 μg/ml G418. Human T cells were purified as described previously (Mueller *et al.*, 1995).

Production of VCAM/Ig fusions

A plasmid containing the human IgG1 Fc region was obtained from Dr M. J. Lenardo (NIH, Bethesda, MD, U.S.A.). DNA fragments containing various domains of human or porcine VCAM were obtained by PCR amplification of appropriate templates and joined in frame to the human IgG1 Fc coding region using standard molecular biology techniques. The cassettes encoding the fusion proteins were ligated into the expression plasmid pAPEX-3 (Evans *et al.*, 1995*a*). VCAM/Ig fusion proteins were produced by transfection of 293-EBNA cells as described previously (Evans *et al.*, 1995*a*). Three days following transfection the media were harvested, centrifuged to remove debris, and stored at 4°C. For ELISA, PolySorp 96-well microtiter plates (Nunc, Naperville, IL, U.S.A.) were coated overnight at 4°C with 100 ng/well goat anti-human IgG Fc (Sigma, St. Louis, MO, U.S.A.) in 0.1 M carbonate buffer, pH 9.6. The plates were then washed three times with PBS supplemented with 0.5% Tween 20 and incubated with blocking buffer (PBS supplemented with 0.05% Tween 20) at 37°C for 1 hr. After three further washes, various dilutions of conditioned media in blocking buffer were added and incubated at 37°C for 1 hr. Following three further washes, 2A2 or 3F4 mAb in blocking buffer was added and incubated for 1 hr at 37°C. After additional washing, peroxidase-conjugated goat anti-mouse IgG Fc (Sigma) diluted 1:2000 in blocking buffer was added and incubated at 37°C for 1 hr. After three final washes the plate was developed for 15 min with 0.4 mg/ml *o*-phenylenediamine dihydrochloride (Sigma). Reactions were stopped by addition of 1 M sulfuric acid. Quantitation was performed by measuring absorbance at 490 nm using a Bio-Rad model 3550 plate reader.

Production of chimeric antibodies

Variable regions were cloned from the 2A2 and 3F4 hybridomas by PCR amplification as described previously (Evans *et al.*, 1995*a*, 1995*b*). These sequences have been submitted to the EMBL/GenBank/DDBL under accession numbers U78799–U78802. The variable regions were joined to genomic DNA cassettes containing

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either the intact human IgG4 constant regions or the first constant region and hinge linker region from human IgG2 followed by the second and third constant regions from human IgG4 using standard molecular biology techniques. Chimeric antibodies were produced in 293-EBNA cells and purified by chromatography on protein A Sepharose as described (Evans *et al.*, 1995*a*). Each chimeric antibody preparation was greater than 95% pure as judged by Coomassie staining of SDS polyacrylamide gels. F(ab')2 was prepared by digestion of the antibodies with ficin (Pierce, Rockford, IL, U.S.A.) followed by protein A chromatography.

Flow cytometry

Cells used were obtained by trypsinization (PAEC and 293-EBNA) or centrifugation (Ramos, Jurkat, YT and U-937 cells). Harvested cells were washed once with 4°C PBS containing 1% heat-inactivated FBS (PBS/1) and aliquoted at 50 000–100 000 cells per well in 96-well V-bottom plates (Nunc). Cells were washed two additional times with PBS/1, and then incubated with PBS/1 containing 10 μg/ml primary Ab or PBS/1 alone for 30 min at 4°C. For some experiments the chimeric HuG4 or HuG2/G4 was centrifuged at $1 \times 10^5 g$ for 1 hr prior to addition to the cells. The cells were then washed twice with PBS/1 and resuspended in PBS/1 containing the appropriate FITC-conjugated secondary antibodies (1:200 dilution) for 30 min at 4°C. The cells were again washed twice with PBS/1 and resuspended in PBS. Cell fluorescence was determined using a Becton Dickinson FACSort.

Adhesion assays

On day 0 PAEC were plated in medium A at a density of 10 000 cells per well in a 96-well flat bottom plate. On day 1 the PAEC were refed medium A supplemented with 1 μg/ml LPS (Sigma, *E. coli* 0111:B4). On day 2, human cells were resuspended in the appropriate growth medium supplemented with 10 μM calcein AM (Molecular Probes, Eugene, OR, U.S.A.) and incubated at 37°C for 30 min. The cells were subsequently washed three times with growth medium and resuspended at 6×10^6 cells/ml in RPMI 1640 supplemented with 1% heat-inactivated FBS (RPMI/1). The PAEC were washed twice with RPMI/1 and refed 50 μl/well of RPMI/1 containing the indicated concentrations of antibodies. After adding calcein-labeled cells at a density of 3×10^5 cells/well (50 μl), the plate was centrifuged at 200*g* for 1 min and incubated at 37°C for 15 min. The nonadherent cells were removed by washing three to five times with RPMI/1. Adherent cells were lysed with HBSS containing 1% SDS and calcein fluorescence was measured with a Millipore CytoFluor 2350 using an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Background fluorescence was determined from wells containing PAEC that did not receive calcein-labeled cells. Samples were performed in duplicate in each experiment. Adherence was determined by comparing the residual fluorescence concentration in each well to the input fluorescence concentration. In each experiment the specific fluorescence signal obtained in the absence of competing antibody was defined as 100%.

For adhesion assays using purified soluble pVCAM (Mueller *et al.*, 1995), PolySorp 96-well microtiter plates (Nunc) were coated overnight at 4°C with 200 ng/well purified soluble pVCAM in 0.1 M carbonate buffer, pH 9.6. After blocking the plate with RPMI 1640 supplemented with 10 mg/ml BSA (RPMI/BSA), the plates were incubated with RPMI/BSA containing antibody for 1 hr at 37°C. Subsequently, 3×10^5 calcein-labeled U-937 cells in RPMI/BSA were added to each well. The plate was centrifuged upright for 1 min at 200*g* and incubated at 37°C for 15 min. The plate was then centrifuged inverted at 200*g* for 3 min. Adherent cells were lysed by the addition of HBSS containing 1% SDS and calcein fluorescence was measured with a Millipore CytoFluor 2350 using an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

RESULTS

Identification of porcine VCAM domains recognized by blocking mAbs

Previously, we have identified two murine mAbs (3F4 and 2A2) directed against porcine VCAM (pVCAM) which block VLA-4 dependent adhesion of peripheral blood human T cells to PAEC. These two mAbs are specific for pVCAM and do not recognize human VCAM (hVCAM). Here an initial analysis of the epitope specificity of the 2A2 and 3F4 mAbs was performed using fusion proteins consisting of various domains of pVCAM or hVCAM joined to the Fc region of human IgG1. The VCAM/Ig fusion proteins were produced in 293-EBNA cells and assayed for binding of 2A2 and 3F4 mAbs by ELISA (Fig. 1). Both the 2A2 and 3F4 mAbs bound efficiently to proteins containing the first three domains of pVCAM. The 2A2 mAb bound to fusion proteins containing pVCAM domain 1 such as pD1 + pD2 (porcine domain 1 plus porcine domain 2) and pD1 + hD2 (porcine domain 1 plus human domain 2), but did not bind to fusion proteins lacking pVCAM domain 1 such as pD2 + pD3, hD1 + pD2, or hD1 + hD2. Weak binding of the 2A2 mAb could also be detected to a fusion protein containing only porcine domain 1 (pD1) but not to fusion proteins containing only pD2 or pD3. Conversely, the 3F4 mAb bound to fusion proteins containing pVCAM domain 2, such as pD1 + pD2, pD2 + pD3, and hD1 + pD2, but did not bind to fusion proteins lacking pVCAM domain 2, such as pD1 + hD2 and hD1 + hD2. Binding of the 3F4 mAb could not be detected to fusion proteins containing only a single pVCAM domain. The weak binding of 2A2 and 3F4 to fusion proteins containing only a single VCAM domain is similar to previously characterized blocking antibodies specific for human VCAM and ICAM-1 which require the presence of at least two adhesion molecule domains for binding (Pepinsky *et al.*, 1992; Renz *et al.*, 1994). Together, these results suggested the 2A2 mAb specificity is defined by

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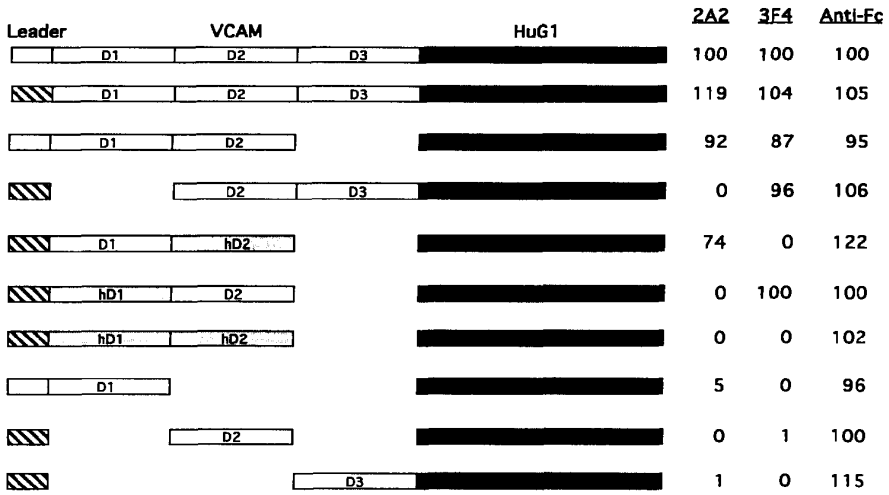


Fig. 1. Binding of 3F4 and 2A2 mAbs to VCAM/Ig fusion proteins. Proteins consisting of domains 1 (D1), 2 (D2), or 3 (D3) from porcine VCAM (open boxes) or human VCAM (gray boxes) joined to the Fc region of human IgG1 (black boxes) were produced in 293-EBNA cells. In some constructs the pVCAM secretory leader sequence was replaced with the secretory leader sequence from human CD59 (striped boxes). The fusion proteins were captured by incubating diluted conditioned media in 96-well plates coated with goat anti-human IgG (Fc specific). After incubation with 2A2 or 3F4 mAb, bound murine mAb was detected using peroxidase-conjugated goat anti-mouse IgG (Fc specific). The total amount of each fusion protein captured per well was quantitated using peroxidase-conjugated goat anti-human IgG (Fc specific). The absorbance signal obtained with the fusion protein containing the intact pVCAM domains 1, 2 and 3 was defined as 100% for each antibody. Binding of the 2A2 and 3F4 mAbs to the remaining fusion proteins is reported as a percentage of this value. Results shown are from a representative experiment of several performed.

pVCAM domain 1 whereas the 3F4 mAb specificity is defined by pVCAM domain 2.

Inhibition of human leukocyte binding to PAEC by chimeric anti-porcine VCAM mAbs

The use of murine antibodies in porcine-to-primate xenotransplantation models or ultimately in porcine-to-human xenotransplants poses several problems including a short half-life of the murine antibody plus development of an immune response directed against the murine antibody. These problems have been significantly reduced by the use of chimeric antibodies consisting of the murine variable regions joined to human constant regions (Khazaeli *et al.*, 1994). To create anti-pVCAM chimeric antibodies we cloned the variable regions from both 2A2 and 3F4. The murine variable heavy regions were joined to the human IgG4 constant region (HuG4) since this isotype does not activate complement (C) (Tao *et al.*, 1993), the predominant mediator of hyperacute rejection of xenografts. The murine variable light regions were joined to the human kappa constant region. Chimeric ch2A2 HuG4 and ch3F4 HuG4 antibodies were produced by coexpression of the chimeric heavy and light chains in 293-EBNA cells and tested for binding to pVCAM by FACS analysis using 293 cells expressing pVCAM (Fig. 2(A)). Both chimeric mAbs produced identical staining profiles as the corresponding murine mAbs, while neither chimeric mAb bound to untransfected 293 cells. Additionally, both the chimeric antibodies and murine mAbs produced identical FACS profiles using LPS-activated porcine aortic endothelial cells (Fig. 2(B)), confirming the chimeric antibodies retained the ability to bind to pVCAM expressed on PAEC.

The ability of the ch2A2 HuG4 and ch3F4 HuG4 antibodies to block the interaction between human VLA-4 and porcine VCAM was assessed by measuring inhibition of Ramos cell binding to activated PAEC (Fig. 3). The murine 3F4 mAb inhibited this binding by 90% when present at concentrations greater than 5 μg/ml. A control humanized antibody directed against human C5 (h5G1.1 HuG4) did not inhibit the binding of Ramos to activated PAEC. The ch2A2 HuG4 and ch3F4 HuG4 produced nearly identical inhibition curves as the murine 3F4 mAb, demonstrating the retention of binding avidity and cell adhesion blocking capabilities in these chimeric antibodies.

The ability of the ch2A2 HuG4 and ch3F4 HuG4 mAbs to inhibit binding of cells expressing both VLA-4 and LFA-1 was next examined using the Jurkat T cell line. Both the ch2A2 HuG4 and ch3F4 HuG4 antibodies inhibited the binding of Jurkat to activated PAEC by up to 90% while the control antibody h5G1.1 HuG4 did not inhibit binding at any concentration (Fig. 4(A)). Similarly, the ch2A2 HuG4 and ch3F4 HuG4 antibodies inhibited the binding of both resting (Fig. 4(B)) and activated (Fig. 4(C)) T cells to activated PAEC, although the

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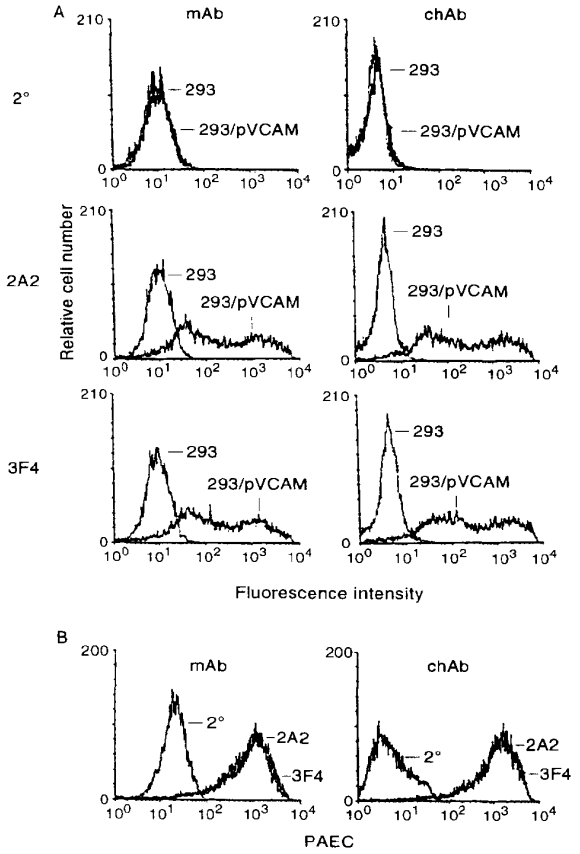


Fig. 2. Binding of 3F4 and 2A2 chimeric antibodies to pVCAM. (A) Chimeric mAbs containing the variable regions of either 2A2 or 3F4 joined to human IgG4 and kappa constant regions were produced in 293-EBNA cells and purified by protein G Sepharose chromatography. Untransfected 293-EBNA cells (293) or 293-EBNA cells expressing pVCAM (293/pVCAM) were incubated with 10 μg/ml purified murine 2A2 mAb, murine 3F4 mAb, ch2A2 HuG4, or ch3F4 HuG4. Bound antibody was detected using either FITC-conjugated goat anti-mouse IgG or FITC-conjugated goat anti-human IgG followed by flow cytometry using a Becton Dickinson FACSort. Data are displayed as histograms with fluorescence intensity plotted on the x-axis and relative cell number plotted on the y-axis. Background staining by the secondary antibodies is shown in the panels labeled 2°. (B) PAEC were activated by treatment with 1 μg/ml LPS for approximately 16 hr. Cells were then stained with either the murine 2A2 and 3F4 mAbs (left panel) or the ch2A2 HuG4 and ch3F4 HuG4 antibodies (right panel). Background staining by the secondary antibodies (2°) is indicated.

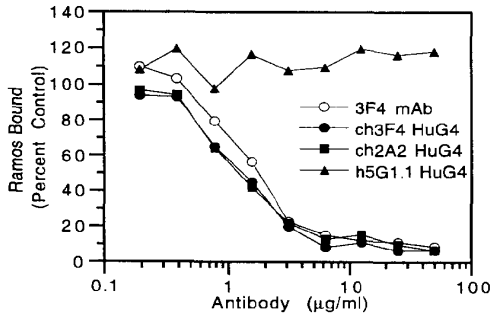


Fig. 3. Binding of Ramos cells to PAEC in the presence of mAbs. On day 0, PAEC were plated at 10 000 cells per well in medium A in 96-well plates. On day 1 the PAEC were activated by adding fresh medium A supplemented with 1 μg/ml LPS. After 16 to 24 hr incubation, the PAEC were washed and refed RPMI 1640 containing 1% FCS plus the indicated concentrations of the murine 3F4 mAb (3F4 mAb), the chimeric 2A2 and 3F4 mAbs (ch2A2 HuG4 and ch3F4 HuG4) or a humanized negative control antibody (h5G1.1 HuG4). Calcein-labeled Ramos cells were added and allowed to adhere to the PAEC under static conditions for 15 min. After nonadherent cells were removed by washing, the remaining bound cells were lysed with SDS. Calcein fluorescence of the SDS supernatant was measured with a Millipore CytoFluor 2350. In each experiment the binding of Ramos cells to PAEC in the absence of antibody was defined as 100%. Results shown are the mean of two to four experiments, with each value determined in duplicate in each experiment.

maximal inhibition achieved was reduced to approximately 60%. Addition of a blocking mAb directed against human VLA-4 also maximally inhibited adhesion by 60% (Fig. 4(C)), confirming the binding inhibition observed was due to blockade of the molecular interaction between human VLA-4 and porcine VCAM.

The potential of the ch2A2 HuG4 and ch3F4 HuG4 antibodies to inhibit binding of natural killer cells and monocytes to activated PAEC was examined using the cell lines YT (Yodio *et al.*, 1985) and U-937 (Sundstron and Nilsson, 1976), respectively. Binding of YT cells to activated PAEC was strongly inhibited by concentrations of 10 μg/ml ch2A2 HuG4 and ch3F4 HuG4 antibodies (Fig. 5(A)). In contrast, the ch2A2 HuG4 and ch3F4 HuG4 antibodies did not inhibit binding of U-937 cells to activated PAEC (Fig. 5(B)). One possible explanation for this failure to inhibit U-937 cell binding could be the presence of elevated levels of VLA-4 on U-937 cells. However, FACS analysis revealed the presence of comparable levels of CD49d and activated CD29 on U-937 cells as on other cell lines such as Jurkat whose adhesion to PAEC was inhibited by the chimeric anti-pVCAM antibodies (Fig. 6). Interestingly, the adhesion of U-937 to PAEC was consistently augmented by approximately 50% in all experiments. This increase was specific for the anti-pVCAM antibodies as no increase in U-937 binding to PAEC was detected in the presence of the control h5G1.1 HuG4 antibody (Fig. 5(B)). This suggested that binding of U-937 to PAEC could be increased through FcR binding to the anti-pVCAM HuG4 antibodies on the PAEC surface. In support of this hypothesis, FACS analysis confirmed the presence of significant levels of FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) on the surface of U-937 cells. In contrast, Jurkat and YT cells had no detectable Fcγ receptors while Ramos cells showed the presence of only low levels of FcγRII (Fig. 6).

Binding of the HuG4 chimeric antibodies to U-937 could be directly demonstrated by flow cytometry (Fig.

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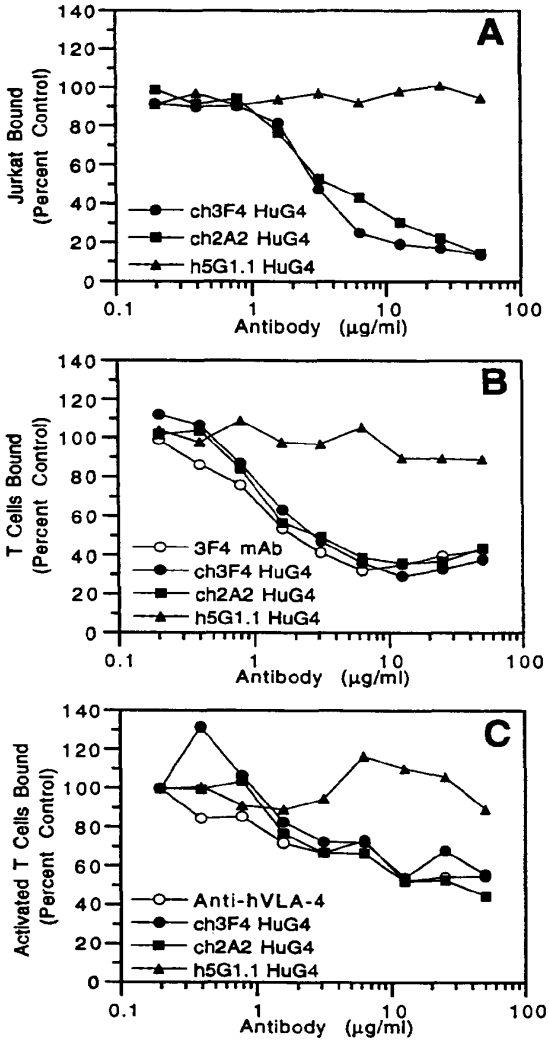


Fig. 4. Binding of Jurkat and human T cells to PAEC in the presence of HuG4 antibodies. Binding experiments were performed as described in Fig. 3 using calcein-labeled Jurkat cells (A) or calcein-labeled purified human T cells (B and C). Human T cells were either assayed immediately after purification (B) or cultured in the presence of 1 μg/ml PHA for 5 days followed by 1 day without PHA stimulation (C) prior to performing the adhesion assay. Results shown are the mean of two to four experiments (A and B) or from an individual experiment (C). Anti-hVLA-4 denotes the blocking murine mAb HP2/1 directed against the α4 subunit of human VLA-4.

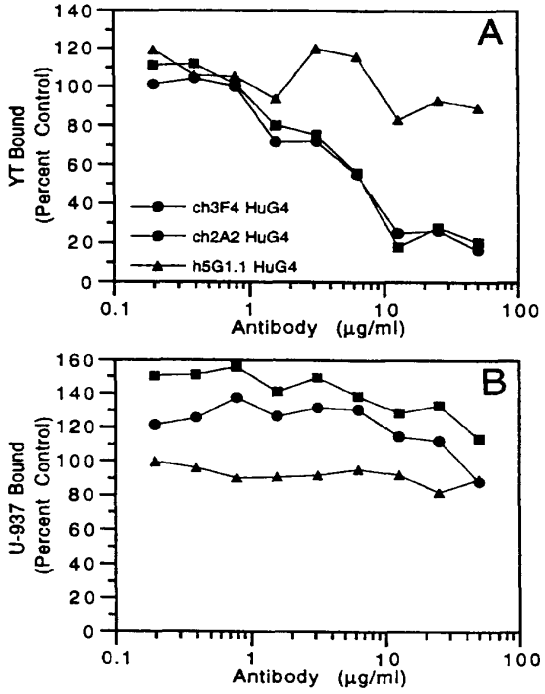


Fig. 5. Binding of YT and U-937 to PAEC in the presence of HuG4 mAbs. Adherence of calcein-labeled YT cells (A) or U-937 cells (B) to PAEC was assayed as described in Fig. 3. Results shown are the mean of two to four experiments.

7). Both the HuG4 antibodies as well as the anti-human C5 HuG4 antibody bound to U-937 cells. In contrast, 293/pVCAM cells bound only the anti-pVCAM antibodies but not the anti-human C5 HuG4 antibody. No binding of the HuG4 chimeric antibodies could be detected with Ramos, Jurkat, or YT cells. These results suggest that the HuG4 chimeric antibodies bound to U-937 cells through interaction of the HuG4 Fc region with the Fc receptors (FcRs) present on U-937 cells. Finally, F(ab')2 fragments prepared from ch3F4 HuG4 strongly inhibited binding of U-937 cells to PAEC (Fig. 8) whereas F(ab')2 fragments prepared from h5G1.1 HuG4 did not inhibit the binding. This result demonstrates that, if antibody crosslinking is prevented, blockade of the interaction between human VLA-4 and porcine VCAM can effectively inhibit the adhesion of human monocytes to PAEC.

Inhibition of FcR binding of humanized mAbs with altered constant regions

Although F(ab')2 fragments proved capable of inhibiting adhesion of U-937 cells, antibody fragments are suboptimal for chronic treatments, as they have much shorter *in vivo* half-lives than intact antibodies (Covell *et al.*, 1986). The use of intact antibodies with human constant regions as anti-rejection agents is complicated by the capacity of such antibodies to fix C and/or to bind FcRs. Human antibodies of the G4 isotype are devoid of C activity (Tao *et al.*, 1993; Smith *et al.*, 1995). Human antibodies of the G2 isotype do not activate C or bind FcRs (Canfield and Morrison, 1991; Burton and Woof, 1992). Rather than altering the specific residues known to mediate C fixation and FcR binding, we explored the possibility of creating an antibody with a composite human constant region. To create chimeric mAbs which would not activate C or bind to FcRs, we utilized the overall similarity in the human G2 and G4 primary

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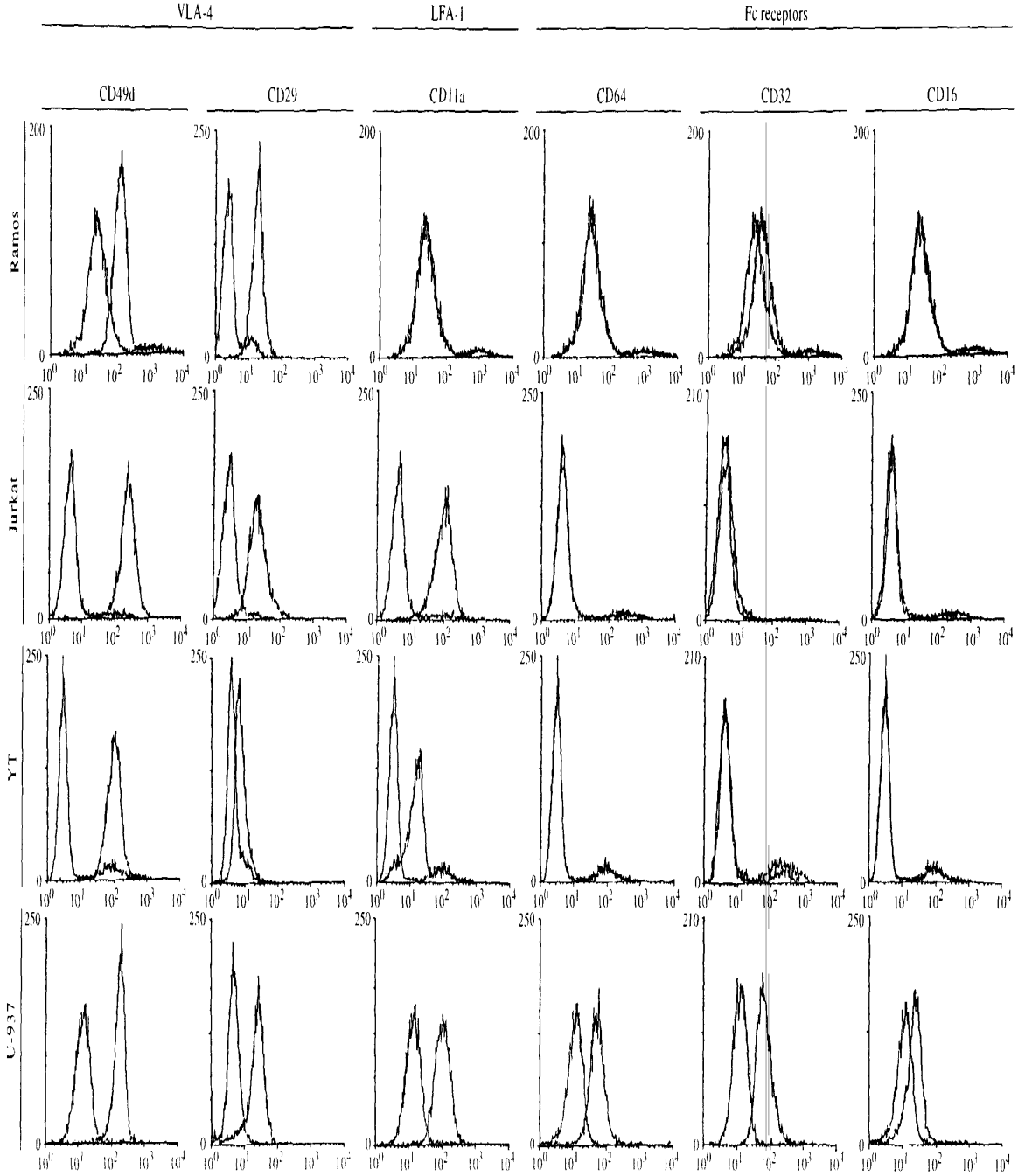


Fig. 6. Expression of VLA-4, LFA-1 and FcRs on the surface of Ramos, Jurkat, YT and U-937 cells. Cells were incubated with 10 μg/ml of the following primary antibodies: mouse anti-human CD49d, rat anti-activated human CD29, mouse anti-human CD11a, mouse anti-human CD64 ($Fc_\gamma RI$), mouse anti-human CD32 ($Fc_\gamma RII$) or mouse anti-human CD16 ($Fc_\gamma RIII$). After washing, cells were incubated with either FITC-conjugated goat anti-mouse IgG or FITC-conjugated goat anti-rat IgG as appropriate. Each panel presents the results obtained in the presence of primary plus secondary antibody or in the presence of secondary antibody alone.

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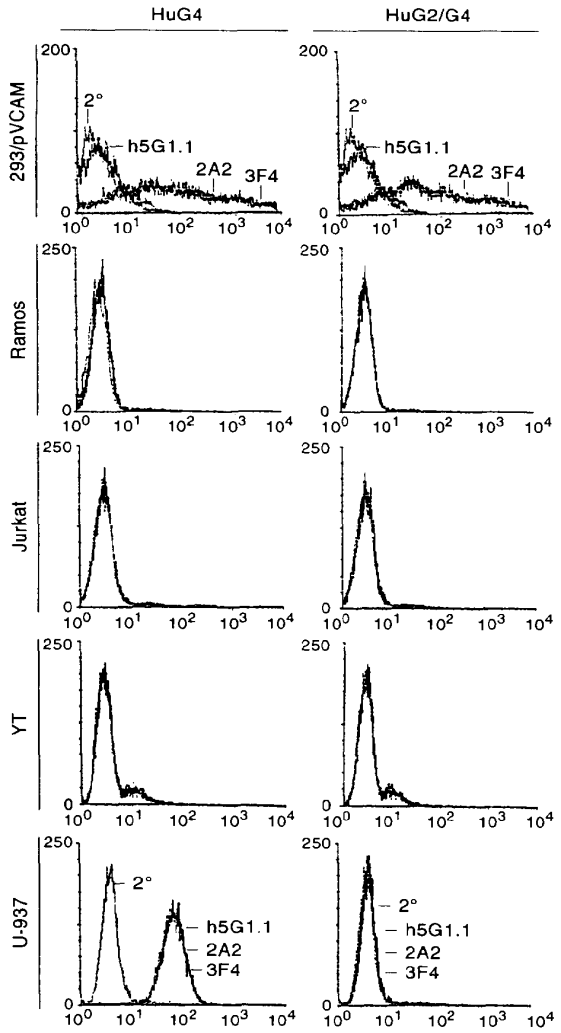


Fig. 7. Binding of chimeric antibodies to 293/pVCAM, Ramos, Jurkat, YT and U-937 cells. Cells were incubated with 10 μg/ml h5G1.1 HuG4, ch2A2 HuG4, or ch3F4 HuG4 (left panels) or 10 μg/ml h5G1.1 HuG2/G4, ch2A2 HuG2/G4, or ch3F4 HuG2/G4 (right panels). After washing, cells were incubated with FITC-conjugated goat anti-human IgG except for Ramos cells which were incubated with FITC-conjugated goat anti-human IgG (γ chain specific). Each panel presents the results obtained with the indicated cell line for h5G1.1 HuG4, ch2A2 HuG4 and ch3F4 HuG4 mAbs as well as the secondary antibody alone (2°) control.

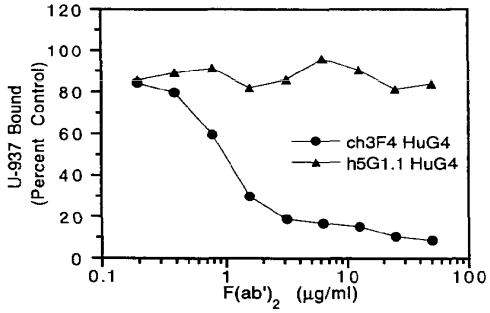


Fig. 8. Binding of U-937 cells to PAEC in the presence of F(ab')2 fragments. F(ab')2 fragments were prepared from h5G1.1 HuG4 and ch3F4 HuG4 by digestion with ficin. Undigested antibody and Fc fragments were removed by protein A chromatography. Adherence of calcein-labeled U-937 cells to PAEC in the presence of F(ab')2 was assayed as described in Fig. 3. Results shown are the mean of five experiments.

sequences to design a HuG2/G4 composite antibody consisting of the first constant region domain and the hinge region from the HuG2 isotype joined to the second and third constant region domains from the HuG4 isotype (Fig. 9). The resulting mAb did not contain the antibody sequences necessary for FcR binding, nor any new epitopes that would likely be immunogenic.

Binding to FcR was examined by using the monocytic cell line U937. FACS analysis demonstrated that the resulting ch3F4 HuG2/G4, ch2A2 HuG2/G4 and h5G1.1 HuG2/G4 antibodies did not bind to U-937 cells (Fig. 7). The ch3F4 HuG2/G4 and ch2A2 HuG2/G4 antibodies maintained the ability to bind pVCAM as demonstrated both by binding to 293/pVCAM cells (Fig. 7) and by inhibition of Jurkat and YT binding to activated PAEC (Fig. 10). For both Jurkat and YT cells inhibition of binding with the chimeric HuG2/G4 was equivalent to the HuG4 or original murine anti-pVCAM antibodies. The binding of U-937 cells to PAEC was now potently inhibited by the presence of either ch2A2 HuG2/G4 or ch3F4 HuG2/G4 antibodies. Finally, the chimeric antibodies were examined for the ability to block the adhesion of U-937 cells to purified soluble pVCAM (Fig. 11). Addition of the ch3F4 HuG4 antibody failed to inhibit binding of U-937 cells to pVCAM unless very high concentrations of antibody were added. In contrast the ch3F4 HuG2/G4 antibody potently inhibited the binding of U-937 cells to pVCAM, while the negative control h5G1.1 HuG2/G4 antibody did not block binding. Together, these results demonstrate the anti-pVCAM HuG2/G4 antibodies specifically inhibited the adhesive interaction of human VLA-4 with porcine VCAM and that disruption of this interaction was sufficient to inhibit binding of numerous human cell types to porcine endothelial cells.

DISCUSSION

Current success in allotransplantation has resulted from the development of effective global immunosuppressive therapies that prevent cellular graft rejection. Recent attempts to develop more specific immunosuppressive strategies have focused on inhibiting interactions between integrins and costimulatory molecules on T cells with their ligands on graft endothelial cells and other antigen presenting cells (reviewed in Faustman, 1995; Guinan *et al.*, 1994). The success of allotransplantation has been limited by the resulting shortage of human donor organs. Recent progress in overcoming the initial

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HuG2      STKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGV
HuG2/G4   STKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGV
HuG4      STKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGV

HuG2      HTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVE
HuG2/G4   HTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVE
HuG4      HTFPAVLQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKVDKRVE

                                235
HuG2      RKCCVECPPCPAPPV-AGPSVFLF
HuG2/G4   RKCCVECPPCPAPPV-AGPSVFLF
HuG4      SKYGPPCPSCPAPEFLGGPSVFLF

HuG2      PPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPR
HuG2/G4   PPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPR
HuG4      PPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPR

                                                      331
HuG2      EEQFNSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTKG
HuG2/G4   EEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKG
HuG4      EEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKG
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Fig. 9. Sequence of the CH1, hinge and CH2 regions of human IgG2 (HuG2), human IgG4 (HuG4), and the chimeric HuG2/G4 construct. Positions where differences occur between the three molecules are double underlined. The amino acid sequence at position 235/236 determines ability to bind to Fcγ receptors (Canfield and Morrison, 1991; Duncan *et al.*, 1988; Alegre *et al.*, 1992; Burton and Woof, 1992) while the presence of a serine at position 331 confers the inability to activate C (Tao *et al.*, 1993).

hyperacute rejection of discordant xenografts suggests the potential for use of porcine xenografts as a clinical option to circumvent this chronic shortage. Although there are limited data regarding the fate of discordant xenografts which survive the initial hyperacute rejection phase, xenograft recipients will likely require continued cellular immunosuppression for the prevention of graft rejection. In this light, xenografts offer a unique opportunity for the development of blocking reagents specific for the graft, thereby avoiding global immunosuppression of the recipient.

To investigate the interactions occurring between human leukocytes and porcine endothelial cells, we have cloned the porcine VCAM molecule and created mAbs specific for porcine VCAM that do not bind to human VCAM (Mueller *et al.*, 1995). Two of these antibodies (3F4 and 2A2) are capable of blocking the interaction between human VLA-4 and porcine VCAM. Here we demonstrate that the binding specificity for 2A2 is located within domain 1 of pVCAM while the binding specificity for 3F4 is located within domain 2. This is identical to the results found with human VCAM, where blocking antibodies have also been shown to be directed against either domains 1 or 2 (Osborn *et al.*, 1992; Vonderheide and Springer, 1992).

The use of murine mAbs in humans and primates presents numerous problems including a short antibody half-life and a high degree of immunogenicity (Khazaeli *et al.*, 1994). To optimize the use of the anti-porcine VCAM antibodies in porcine-to-primate xenograft experiments we developed chimeric antibodies using the variable regions cloned from the 2A2 and 3F4 antibodies joined to the constant regions of the human IgG4,κ isotype. Given the extensive activation of C which occurs within xenografts, the human IgG4 isotype was chosen due to its inability to activate C. The resultant chimeric antibodies (ch2A2 HuG4 and ch3F4 HuG4 antibodies) retained specificity for porcine VCAM and were as potent as the original murine mAbs at blocking adhesion of Ramos cells to activated PAEC by greater than 90% (Fig. 3). In contrast to Ramos, the binding of resting or activated human T cells to activated PAEC was inhibited only approximately 50% by either the chimeric antibodies or the original mAbs (Fig. 4). Activation of human T cells by PAEC can be blocked by antibodies directed against either CD2 or LFA-1 (Rollins *et al.*, 1994*b*), suggesting either of these molecules could mediate the remaining adhesion by interacting with porcine CD58 or porcine ICAM-1, respectively.

The adhesion of human lymphocytes, monocytes or NK cells to activated HUVEC is dependent on the interaction of both LFA-1 with ICAM-1 and VLA-4 with VCAM (Allavena *et al.*, 1991; Jonjic *et al.*, 1992; Vonderheide and Springer, 1992). For each cell type, blocking the VLA-4/VCAM interaction by itself produces only a small reduction in adhesion, while antibodies which disrupt the LFA-1/ICAM-1 interaction provide a greater, although still partial, inhibition of adhesion. Addition of antibodies blocking both VLA-4/VCAM and LFA-1/ICAM-1 is required to completely block adhesion of these cell types to HUVEC. Surprisingly, binding of LFA-1$^+$–VLA$^+$4 Jurkat or YT cells to activated PAEC could be inhibited nearly completely by the ch3F4 HuG4 and ch2A2 HuG4 antibodies. One possible explanation for this could be the lack of interaction between human LFA-1 and porcine ICAM. The conservation of ICAM-1 is relatively low between species, with human and mouse ICAM-1 being only 50% conserved at the amino acid level (reviewed in Carlos and Harlan, 1994), and *in vitro* mouse LFA-1 does not bind to human ICAM-1. Alternatively, human LFA-1 may interact with porcine ICAM-1, but with a reduced affinity. For example, human VLA-4 binds to human VCAM at two independent sites, one within domain 1 and one within

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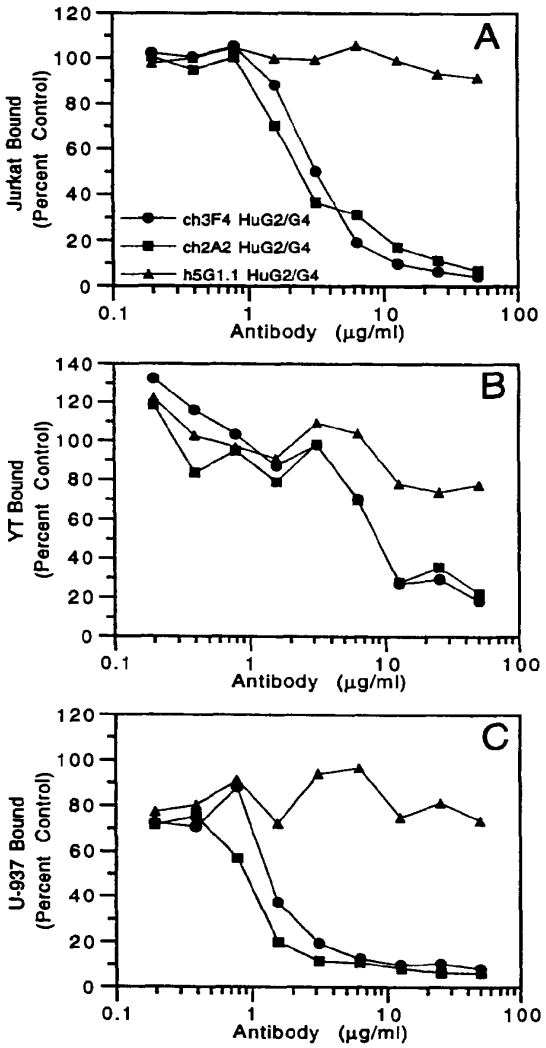


Fig. 10. Binding of Jurkat, YT and U-937 to PAEC in the presence of HuG2/G4 mAbs. Adherence of calcein-labeled Jurkat (A), YT (B) and U-937 (C) cells to PAEC was assayed as described in Fig. 3. Results shown are the mean of two to four experiments.

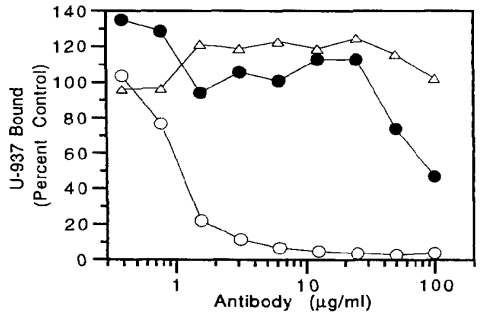


Fig. 11. Binding of U-937 to purified pVCAM. Microtiter plate wells were coated with purified soluble pVCAM. After blocking with FCS, wells received the indicated concentrations of either ch3F4 HuG4 (●), ch3F4 HuG2/G4 (○) or h5G1.1 HuG2/G4 (△) followed by 3×10^5 calcein-labeled U-937 cells. The cells were allowed to adhere at 37°C for 15 min after which non-adherent cells were removed by washing. Adherence of U-937 cells in the absence of competitor antibody was defined as 100%. Results shown are from an individual representative experiment.

domain 4 (Osborn *et al.*, 1992; Vonderheide *et al.*, 1994). In contrast, porcine VCAM lacks domain 4, suggesting the interaction between human VLA-4 and porcine VCAM may be weaker than the interaction between human VLA-4 and human VCAM. Reduced ligand affinities could make the binding of human effector cells to porcine endothelial cells more susceptible to disruption of any one of multiple weak interactions. These observations suggest the potential for significant differences in the therapeutic efficacy achieved by inhibiting particular receptor–ligand interactions with xenografts as compared to allografts.

Although the ch3F4 HuG4 and ch2A2HuG4 blocked binding of Jurkat and YT to activated PAEC, both antibodies failed to block binding of the monocytic U-937 cells to activated PAEC (Fig. 5). The levels of activated $\beta 1$ were comparable on Jurkat and U937 cells indicating an increased level of functional VLA-4 was not the mechanism behind the lack of inhibition of U-937 binding. Additionally, flow cytometry demonstrated the direct binding of the chimeric HuG4 antibodies to U-937 cells (Fig. 7). This binding was not dependent on the antigen specificity of the variable region, suggesting the interaction occurred through the Fc portion of the antibody. Finally, F(ab′)2 prepared from 3F4 HuG4 were able to block the adhesion of U-937 cells to activated PAEC by greater than 80% (Fig. 8). Together these results were consistent with a model in which the chimeric anti-pVCAM HuG4 mAbs crosslinked U-937 cells to activated PAEC by simultaneously binding FcRs and cell surface VCAM present on the PAEC. This is comparable to the situation encountered with the anti-CD3 murine monoclonal antibody (mAb) OKT3, which when first administered to patients produces crosslinking of T cells to FcR bearing cells, resulting in the activation of both cell types (Ceuppens *et al.*, 1985; Kan *et al.*, 1986) with resultant toxicity to the patient.

The interaction of antibodies with Fcγ receptors has been found to be dependent on the sequence of the hinge linker region of the CH2 domain (Duncan *et al.*, 1988; Alegre *et al.*, 1992; Burton and Woof, 1992) as well as an additional bend located between two β strands within the CH2 domain (Canfield and Morrison, 1991). In the case of a humanized OKT3 antibody (human IgG4 constant regions), binding to Fcγ receptors has been eliminated by the introduction of amino acid mutations in the hinge linker (Alegre *et al.*, 1992, 1994). In either case the resulting human IgG4 constant region contains amino acid differences which could be immunogenic. Here, we have created chimeric antibodies (designated HuG2/G4) containing the CH1 domain and hinge linker region of

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human IgG2 followed by the CH2 and CH3 domains of the human IgG4 constant region. Due to the similarities between these two isotypes at the amino acid level, the resultant antibody contained no novel peptides less than 30 amino acids in length likely to be presented by MHC class II molecules and invoke an immune response. The chimeric HuG2/G4 antibodies no longer bound to U-937 cells. Moreover, the ch2A2 HuG2/G4 and ch3F4 HuG2/G4 antibodies blocked the binding of Jurkat and YT cells to activated PAEC as potently as the chimeric HuG4 antibodies, demonstrating the alteration of the Fc region did not reduce the antibody avidities. Moreover, the chimeric anti-pVCAM HuG2/G4 recombinant antibodies nearly completely blocked the binding of U-937 cells to activated PAEC and to purified pVCAM. Finally, as predicted from the low affinity of IgG2 and IgG4 for human C1q, our preliminary results indicate that the HuG4 and HuG2/G4 recombinant antibodies show little or no C1q binding (data not shown).

In summary, we have developed humanized mAbs specific for porcine VCAM which potently block the adhesion of multiple human inflammatory cell types to porcine endothelial cells. The lack of binding of these antibodies to human VCAM leaves intact the interaction of human leukocytes with human endothelial cells. The adhesion-blocking properties of these antibodies will allow delineation of the role of VLA-4/VCAM interactions in cellular mediated rejection of porcine-to-primate xenografts. Additionally, the HuG2/G4 antibody design should prove useful in humanization of other antibodies intended for human use where elimination of FcR binding and C activation may be desirable.

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ORIGINAL ARTICLE

The Complement Inhibitor Eculizumab in Paroxysmal Nocturnal Hemoglobinuria

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ABSTRACT

BACKGROUND

We tested the safety and efficacy of eculizumab, a humanized monoclonal antibody against terminal complement protein C5 that inhibits terminal complement activation, in patients with paroxysmal nocturnal hemoglobinuria (PNH).

METHODS

We conducted a double-blind, randomized, placebo-controlled, multicenter, phase 3 trial. Patients 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 other week through week 26. The two primary end points were the stabilization of hemoglobin levels and the number of units of packed red cells transfused. Biochemical indicators of intravascular hemolysis and the patients' quality of life were also assessed.

RESULTS

Eighty-seven patients underwent randomization. Stabilization of hemoglobin levels in the absence of transfusions was achieved in 49% (21 of 43) of the patients assigned to eculizumab and none (0 of 44) of those assigned to placebo (P<0.001). 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<0.001). Eculizumab reduced intravascular hemolysis, as shown by the 85.8% lower median area under the curve for lactate dehydrogenase plotted against time (in days) in the eculizumab group, as compared with the placebo group (58,587 vs. 411,822 U per liter; P<0.001). Clinically significant improvements were also found in the quality of life, as measured by scores on the Functional Assessment of Chronic Illness Therapy-Fatigue instrument (P<0.001) and the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire. Of the 87 patients, 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.

CONCLUSIONS

Eculizumab is an effective therapy for PNH. (ClinicalTrials.gov number, NCT00122330.)

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PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (PNH), an uncommon form of hemolytic anemia, results from the clonal expansion of hematopoietic stem cells that have somatic mutations in the X-linked gene *PIG-A*.[1,2] *PIG-A* mutations cause an early block in the synthesis of glycosylphosphatidylinositol (GPI) anchors, which tether many proteins to the cell surface. Consequently, the blood cells in patients with PNH have a partial deficiency (type II) or a complete deficiency (type III) of GPI-linked proteins.

Intravascular hemolysis is a prominent feature of PNH and is the consequence of the absence of the GPI-linked complement regulatory protein CD59.[3,4] CD59 blocks the formation of the terminal complement complex (also called the membrane-attack complex) on the cell surface, thereby preventing erythrocyte lysis and in vitro platelet activation.[5-8] Excessive or persistent intravascular hemolysis in patients with PNH causes anemia, hemoglobinuria, and complications related to the presence of plasma free hemoglobin, including thrombosis, abdominal pain, dysphagia, erectile dysfunction, and pulmonary hypertension.[9-12] Indeed, the symptoms in PNH are often disproportionate to the degree of anemia. Many patients with this disease are dependent on transfusions. Currently, there is no therapy that effectively reduces intravascular hemolysis or improves the symptoms in patients with PNH.

Eculizumab (Soliris, Alexion Pharmaceuticals) is a humanized monoclonal antibody directed against the terminal complement protein C5.[13] In a preliminary, 12-week, open-label clinical study involving 11 patients with PNH, eculizumab reduced intravascular hemolysis and the patients' transfusion requirements.[14] However, this two-center, uncontrolled study did not have a control group or predefined criteria for the administration of a transfusion, such as a predefined hemoglobin level at which transfusions were administered or a prespecified number of units of packed red cells for a given hemoglobin level.

We report the results of the phase 3 Transfusion Reduction Efficacy and Safety Clinical Investigation, a Randomized, Multicenter, Double-Blind, Placebo-Controlled, Using Eculizumab in Paroxysmal Nocturnal Hemoglobinuria (TRIUMPH) study, which investigated whether eculizumab stabilized hemoglobin levels and reduced transfusion requirements in 87 transfusion-dependent patients with PNH during 6 months of treatment. Intravascular hemolysis and the quality of life were also assessed.

METHODS

PATIENTS

The trial consisted of a 2-week screening period, an observation period of up to 3 months, and a 26-week treatment period. Patients 18 years of age or older who had received at least four transfusions during the previous 12 months were eligible. A PNH type III erythrocyte proportion of 10% or more, platelet counts of at least 100,000 per cubic millimeter, and lactate dehydrogenase levels that were at least 1.5 times the upper limit of the normal range were also required. Concomitant administration of erythropoietin, immunosuppressive drugs, corticosteroids, coumarins, low-molecular-weight heparins, iron supplements, and folic acid were permitted, provided that the doses were constant before and throughout the study. Because persons who have a genetic deficiency of terminal complement proteins have an increased risk of neisserial infections, all patients were vaccinated against *Neisseria meningitidis* with the use of locally approved vaccines. The protocol was approved by the institutional review board at each center, and all patients gave written informed consent.

Patients receiving transfusions who had a mean hemoglobin level greater than 10.5 g per deciliter before transfusion during the 12 months before entry into the study or who had received another investigational drug within 30 days before the first visit were excluded. Patients who had a complement deficiency, an active bacterial infection, or a history of meningococcal disease and those who had undergone bone marrow transplantation were also excluded.

An individualized transfusion algorithm was calculated for each patient on the basis of the history of transfusions during the previous 12 months; the written algorithm documented the number of units of packed red cells to be transfused for given hemoglobin values and served as a prospectively determined guide for transfusion during the observation and treatment periods. Before randomization, eligible patients were observed for up to 13 weeks. Patients who did not require a transfusion during the observation period were considered ineligible. A transfusion administered to a patient who had a hemoglobin

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level of 9 g per deciliter or less with symptoms or 7 g per deciliter or less with or without symptoms qualified the patient for the study (qualifying transfusion) and established the hemoglobin set point. This set point was required for the primary efficacy variable and was individualized for each patient.

STUDY DESIGN

Randomization was performed centrally in a 1:1 ratio without blocking and with stratification according to the number of units of packed red cells transfused during the past year; patients were assigned, by means of an interactive voice–response system, to receive either placebo or eculizumab within 10 days after the administration of the qualifying transfusion. Patients received infusions of 600 mg of eculizumab or placebo every week (±2 days) for 4 weeks, followed 1 week (±2 days) later by 900 mg of eculizumab or placebo, and then by a maintenance dose of 900 mg of eculizumab or placebo every 2 weeks (±2 days) through week 26.

CLINICAL EFFICACY

The two primary end points were the stabilization of hemoglobin levels, defined as a hemoglobin value that was maintained above the level at which the qualifying transfusion was administered, in the absence of transfusions during the 26-week treatment period, and the number of units of packed red cells transfused during that period. The trigger for the administration of transfusions during the study remained unchanged: patients received transfusions when they had symptoms resulting from anemia and their hemoglobin levels reached the individualized, predetermined set point. Prespecified secondary end points included transfusion independence; hemolysis, as measured by the lactate dehydrogenase value for the area under the curve from baseline to 26 weeks; and changes in the level of fatigue, as assessed from baseline to 26 weeks with the use of the Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT-Fatigue) instrument (scores can range from 0 to 52, with higher scores indicating improvement in fatigue).[15] Prespecified exploratory analyses included assessment of the quality of life with the use of the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire (EORTC QLQ-C30) (scores can range from 0 to 100, with higher scores on the global health status and functioning scales and lower scores on the symptom scales and single-item measures indicating improvement)[16]; changes in lactate dehydrogenase levels from baseline through week 26; and the presence of thrombosis. Other prespecified measurements included the pharmacokinetics, pharmacodynamics, and immunogenicity of eculizumab. The time to the first transfusion during the treatment period and the proportion of PNH type III blood cells were assessed.

SAFETY

Adverse events related to study infusions and vital signs (assessed at each of the 17 study visits during treatment), the results of biochemical analyses and blood counts (assessed at 9 visits), and findings on electrocardiograms (assessed at 3 visits) were documented. Adverse events were coded with the use of preferred terms from the Medical Dictionary for Regulatory Activities (MedDRA) (www.msso.org/MSSOWeb/index.htm) and tabulated as incidence rates in the two study groups.

STATISTICAL ANALYSIS

The planned sample size of 75 patients provided the study with a statistical power of 82%, at an alpha level of 0.05, to detect an increase of 35 percentage points (i.e., a change from 20% to 55%) in the rate of the stabilization of hemoglobin levels and a reduction in the median number of units of packed red cells transfused from 6 to 2 (±2). For the two primary end points, the analyses were performed according to the intention-to-treat principle with the use of data on all 87 patients who underwent randomization; stabilization of hemoglobin levels was analyzed with the use of Fisher's exact test, and the total number of units of packed red cells transfused was analyzed with the use of the Wilcoxon rank-sum test. To assess the effect of treatment on whether or not transfusions were required, Fisher's exact test was used. The log-rank test was used to compare the time to the first transfusion in the two groups. The area under the curve for lactate dehydrogenase was compared between the two groups with the use of the Wilcoxon rank-sum test.

Fatigue was assessed according to the scoring guidelines for the FACIT-Fatigue instrument.[17] The assessment of the quality of life was based on the EORTC QLQ-C30 scores and was conducted as described previously.[18] Changes in scores

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on the FACIT-Fatigue instrument and the EORTC QLQ-C30 instrument from baseline through week 26 were analyzed with the use of a mixed model, with baseline scores as the covariate, treatment and time as fixed effects, and the patient identifier as a random effect. Changes in the levels of lactate dehydrogenase, PNH type III erythrocytes, and hemoglobin from baseline through week 26 were analyzed with the use of the same mixed model. All reported P values are two-sided and were not adjusted for multiple analyses. The incidence rates of adverse events were compared with the use of Fisher's exact test. No interim analyses were performed, and blinding regarding the results was maintained until the end of the study.

The authors and the sponsor were jointly responsible for the trial design and the development of the protocol. Data were collected by an electronic case-report form with the use of InForm software (version 4.0, Phase Forward) and were analyzed by the sponsor. The decision to publish the trial data and final decisions on the content of the manuscript rested with Dr. Hillmen in consultation with the other authors. The manuscript was prepared by Dr. Hillmen, with substantial review and comments by the other authors. All authors had access to the primary data and take responsibility for the veracity and completeness of the data reported.

Table 1. **Baseline Characteristics of the Patients.**

Characteristic	Placebo Group (N=44)	Eculizumab Group (N=43)
Sex — no.		
Male	15	20
Female	29	23
Age — yr		
Median	35	41
Range	18–78	20–85
Duration of PNH — yr		
Median	9.2	4.3
Range	0.5–38.5	0.9–29.8
Reticulocyte counts — per mm^3		
Median	204,400	206,600
Range	45,400–556,200	40,200–570,400
History of aplastic anemia — no. (%)	12 (27)	6 (14)
History of myelodysplastic syndrome — no. (%)	0	2 (5)
History of thrombosis — no. (%)	8 (18)	9 (21)
Total no. of thrombotic events	11	16
Use of erythropoietin — no. (%)	0	3 (7)
Use of cyclosporine — no. (%)	1 (2)	1 (2)
Use of anticoagulant agents (coumarins or heparins) — no. (%)	11 (25)	21 (49)
Use of corticosteroids or androgenic steroids — no. (%)	12 (27)	12 (28)

RESULTS

PATIENTS' CHARACTERISTICS

Of a total of 115 patients with PNH who underwent screening, 87 (35 men and 52 women) at 34 sites in the United States, Canada, Europe, and Australia who received a qualifying transfusion, met the inclusion criteria and did not meet any of the exclusion criteria were randomly assigned to eculizumab (43 patients) or placebo (44 patients) between October 2004 and June 2005. At each of 16 sites one patient underwent randomization, at each of 6 sites two patients underwent randomization, and at each of 12 sites 3 or more patients underwent randomization. There were no significant differences in the baseline characteristics of the patients in the two groups (Table 1).

Of 87 patients who underwent randomization, 85 completed the trial (see the Supplementary Appendix, available with the full text of this article at www.nejm.org). Two patients in the eculizumab group did not complete the trial, one because traveling to the study center was inconvenient and the other because of pregnancy; these patients were included in the analyses. Ten patients in the placebo group discontinued infusions because of a perceived lack of efficacy but remained in the study for monitoring, as prespecified in the protocol, and were included in the analyses.

PHARMACOKINETICS AND PHARMACODYNAMICS

In 42 of 43 patients in the eculizumab group, a 900-mg dose of eculizumab every 2 weeks (±2 days) completely blocked serum hemolytic activity, as assessed by a presensitized erythrocyte hemolytic assay,[14] throughout the study period. In one patient, therapeutic trough levels of eculizumab were not maintained.

EFFECT ON HEMOLYSIS

The effect of eculizumab on chronic intravascular hemolysis was demonstrated by an immediate

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(1 week) and sustained decrease in lactate dehydrogenase levels (Fig. 1A). In the eculizumab group, the mean (±SE) lactate dehydrogenase level decreased from 2199.7±157.7 U per liter at baseline to 327.3±67.6 U per liter at 26 weeks, whereas in the placebo group the levels remained elevated, with values of 2258.0±154.8 U per liter at baseline and 2418.9±140.3 U per liter at 26

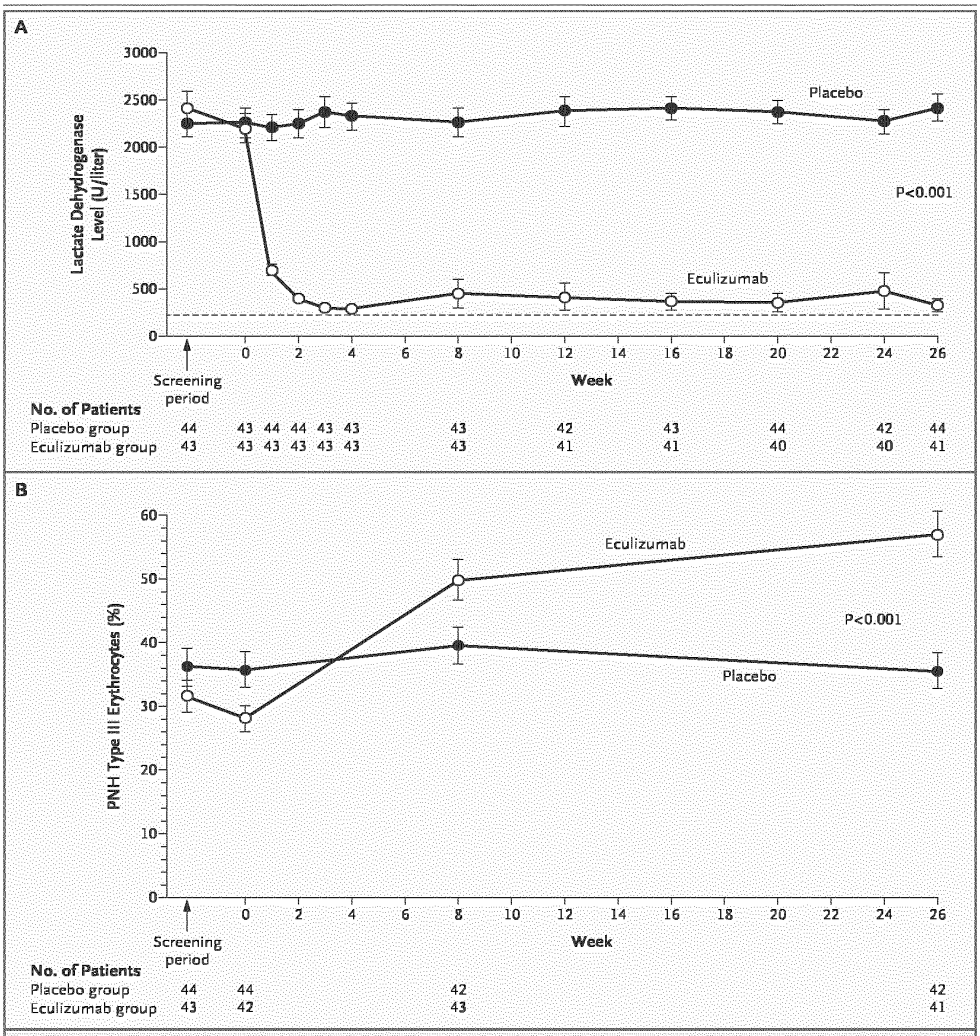


Figure 1. **Levels of Lactate Dehydrogenase and PNH Type III Erythrocytes during Treatment with Eculizumab.**
Panel A shows the degree of intravascular hemolysis according to the mean levels of lactate dehydrogenase from baseline (week 0) to week 26 in the two study groups. The dashed line indicates the upper limit of the normal range for lactate dehydrogenase (normal range, 103 to 223 U per liter). In the eculizumab group the mean level of lactate dehydrogenase was reduced to just above the upper limit of the normal range at week 26; of 41 patients in this group who completed the study, 15 had levels within the normal range. In the placebo group, all patients had levels at least five times above the upper limit of normal at week 26. Panel B shows the mean proportion of PNH type III erythrocytes in patients in the two groups. Screening occurred up to 3 months before week 0. P values are from a mixed analysis-of-covariance model from baseline through week 26. I bars indicate the standard error.

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weeks (P<0.001). The median value of the areas under the curve for lactate dehydrogenase plotted against time (in days) was 85.8% lower in the eculizumab group than in the placebo group (58,587 vs. 411,822 U per liter; P<0.001). A second biochemical measure of hemolysis, the serum level of aspartate aminotransferase, also showed significant improvement with eculizumab, as compared with placebo (data not shown).

The reduction in intravascular hemolysis in the eculizumab group resulted in an increase in PNH type III erythrocytes (Fig. 1B) from a mean of 28.1±2.0% at baseline to 56.9±3.6% at week 26. The proportion of PNH type III erythrocytes in patients in the placebo group remained constant (35.7±2.8% before treatment and 35.5±2.8% at 26 weeks, P<0.001 for the comparison with the eculizumab group and the placebo group). The proportion of PNH type III granulocytes and monocytes did not change significantly between the two groups (data not shown).

CLINICAL EFFICACY

Primary End Points

The two primary efficacy end points were the stabilization of hemoglobin levels and the number of units of packed red cells transfused. At the end of the treatment period, 49% of patients in the eculizumab group (21 of 43) had levels of hemoglobin that remained above the prespecified set point (median, 7.7 g per deciliter for both groups) in the absence of transfusions, whereas stabilization of hemoglobin levels did not occur in any patient in the placebo group (P<0.001) (Table 2). By week 26, the median number of units of packed red cells transfused per patient was 0 in the eculizumab group and 10 in the placebo group (P<0.001), whereas the mean number of units of packed red cells transfused was 3.0±0.7 and 11.0±0.8, respectively. In the 6-month period before the study, the median number of units of packed red cells transfused per patient was 9.0 in the eculizumab cohort and 8.5 in the placebo cohort, and the mean number of units of packed red cells transfused was 9.6±0.6 and 9.7±0.7, respectively. The mean hemoglobin levels changed from 10.0±0.2 g per deciliter and 9.7±0.2 g per deciliter in the eculizumab group and the placebo group, respectively, at baseline to 10.1±0.2 g per deciliter and 8.9±0.2 g per deciliter, respectively, at week 26 (P<0.001, by mixed-model analysis).

The median time to the first transfusion was significantly longer in eculizumab-treated patients than in patients who received placebo (P<0.001) (Fig. 2). Transfusion independence was achieved in 51% of patients in the eculizumab group (22 of 43) and 0% of those in the placebo group (0 of 44, P<0.001). By week 26, the total number of units of packed red cells transfused was 131 in the eculizumab group and 482 in the placebo group (Table 2). By contrast, during the 6 months before the study, the total number of units transfused was 413 in the eculizumab group and 417 in the placebo group.

Table 2. Stabilization of Hemoglobin Levels and the Number of Units of Packed Red Cells Transfused during Treatment.*

Primary End Point	Before Treatment†		During Treatment		P Value
	Placebo Group	Eculizumab Group	Placebo Group	Eculizumab Group	
Patients with stabilized hemoglobin levels (%)	NA	NA	0	49	<0.001‡
Packed red cells transfused (units/patient)					
Median	8.5	9.0	10	0	<0.001§
Interquartile range	7–12.5	6–12	6–16	0–6	
Mean	9.7±0.7	9.6±0.6	11.0±0.8	3.0±0.7	
Total	417	413	482	131	

* Plus–minus values are means ±SE. NA denotes not applicable.
† Transfusion data obtained during 12 months before treatment were normalized to a value equivalent to the value for a 6-month period.
‡ The P value is for the comparison between groups during treatment, calculated with the use of a two-tailed Fisher's exact test.
§ The P value is for the comparison between groups during treatment, calculated with the use of the Wilcoxon rank-sum test.

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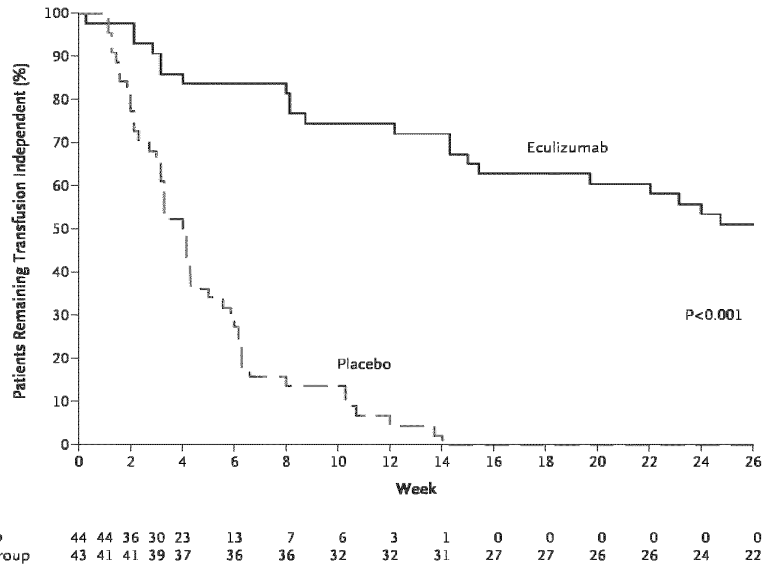


Figure 2. **Kaplan–Meier Curves for the Time to the First Transfusion during Treatment.**
The P value for the comparison of times to the first transfusion between the two groups was calculated by the log-rank test.

Quality of Life

Assessments of the quality of life were performed with the use of two instruments, the FACIT-Fatigue instrument and the EORTC QLQ-C30 instrument. Patients in the eculizumab group had a mean increase (improvement) in scores on the FACIT-Fatigue instrument of 6.4±1.2 points from baseline to week 26, whereas in the placebo group the mean score decreased by 4.0±1.7 points during this period, for a total difference between the two groups of 10.4 points (Fig. 3). A mixed-model analysis of covariance was performed that showed a significant difference between the two groups ($P<0.001$).

With respect to the EORTC QLQ-C30 instrument, the eculizumab group had significant improvements in scores on the scale for global health status, on all five scales for functioning, on two of three symptom scales, and on three of six single-item measures, as compared with the placebo group ($P\leq0.01$ for each scale and measure) (Table 3).

SAFETY

No patients died during the study. Serious adverse events were reported in 13 patients: 4 in the eculizumab group and 9 in the placebo group (Table 4). No serious adverse events 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. The number of headaches that occurred was similar in the two groups after the first 2 weeks of therapy. There were no significant differences in the incidence rates between the two groups for any reported adverse event. A single thrombosis occurred in a patient in the placebo group.

One patient in each of the two groups had detectable levels of antibodies against eculizumab. The levels were low, were detected at a single visit, and in the patient receiving eculizumab, the antibodies did not affect complement inhibition.

DISCUSSION

Patients with PNH have chronic intravascular hemolysis with acute exacerbations. Anemia and the

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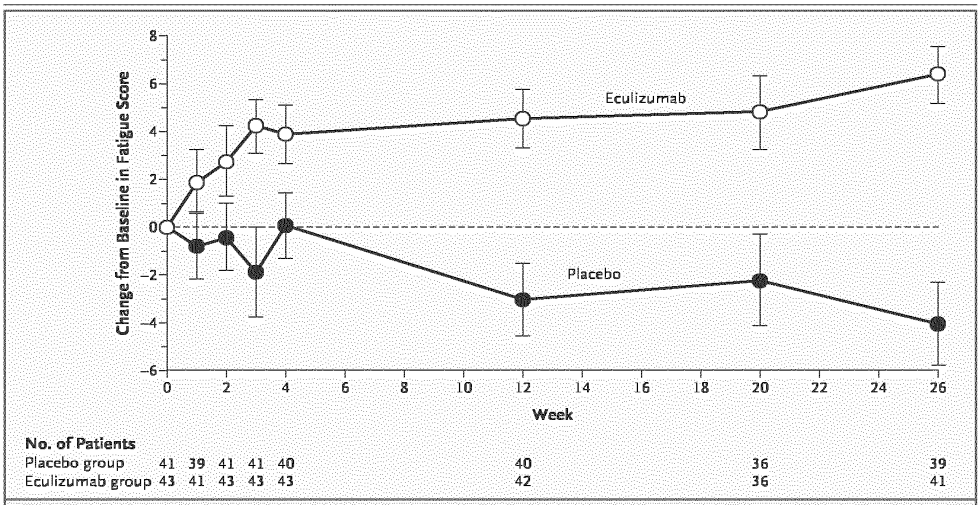


Figure 3. Change in Fatigue Scores from Baseline to Week 26.

Week 0 (baseline) represents the end of the observation period, within 10 days after the patient's receipt of the qualifying blood transfusion. Fatigue scores are therefore higher (indicating less fatigue) at week 0 than at the initial screening visits (data not shown). Values for the change from baseline (dashed line) represent means. A positive change from baseline indicates an improvement in fatigue and a negative change from baseline indicates a worsening in fatigue. I bars indicate the standard error.

need for transfusions to sustain hemoglobin levels occur frequently, as does deterioration of the patient's quality of life. In this study, in approximately half the patients treated with eculizumab, the end points of stabilization of hemoglobin levels and transfusion independence were reached, whereas none of the patients in the placebo group reached either of these end points. The median time to the first transfusion was 4 weeks in the placebo group and more than 6 months in the eculizumab group. The overall rate of transfusion was reduced by 73% in the eculizumab group. Even among patients receiving eculizumab in whom transfusion independence was not reached, the number of units of packed red cells transfused was reduced by 44%, as compared with patients in the placebo group (data not shown).

Intravascular hemolysis is central to the occurrence of serious coexisting conditions in patients with PNH and contributes to the risk of death among these patients.[9,12] Lactate dehydrogenase, a biochemical marker of hemolysis, was immediately reduced from approximately 10 times the upper limit of the normal range to normal levels or to just above normal levels in all patients in the eculizumab group. Residual low-level hemolysis in some patients despite terminal-complement blockade may be caused by an inherent decrease in the survival of PNH type III erythrocytes[19] or may be due to the fact that these cells are opsonized with C3b, which mediates extravascular clearance through the reticuloendothelial system.

Before treatment with eculizumab, the hemoglobin levels were maintained by transfusion. Therefore, the stabilization of hemoglobin levels with a concomitant cessation of or reduction in the number of transfusions indicates an increase in endogenous erythrocyte mass. The reduction in hemolysis with eculizumab results in a new steady-state hemoglobin level, as determined by a balance of the underlying bone marrow dysfunction, the increased half-life of PNH erythrocytes because of eculizumab therapy, and the new level of transfusions (if any) required.

For most patients with PNH, the quality of life is impaired, and the impairment has been attributed not only to anemia but also to excessive in-

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Table 3. Change in the Quality of Life during Treatment.*

Scale	Mean Change in Score from Baseline to Week 26†		Absolute Difference	P Value‡
	Placebo Group	Eculizumab Group		
Global health status scale	−8.5	10.9	19.4	<0.001
Functioning scales				
Role	−6.9	17.9	24.8	<0.001
Social	2.0	16.7	14.7	0.003
Cognitive	−6.1	7.9	14.0	0.002
Physical	−3.5	9.4	12.9	<0.001
Emotional	−3.7	7.5	11.2	0.008
Symptom scales				
Fatigue	10.0	−16.9	26.9	<0.001
Pain	5.3	−12.3	17.6	0.002
Nausea and vomiting	2.8	−0.4	3.2	0.06
Single-item measures				
Dyspnea	8.9	−7.9	16.8	<0.001
Loss of appetite	3.3	−10.3	13.6	<0.001
Insomnia	4.9	−7.9	12.8	0.01
Financial difficulties	0.0	−10.3	10.3	0.19
Constipation	0.0	−6.3	6.3	0.20
Diarrhea	5.7	4.8	0.9	0.15

* The quality of life was assessed with the EORTC QLQ-C30 instrument.
† A positive value for a score on the scales for global health status and functioning indicates improvement, whereas a negative value for a score on the symptom scales and for a score on the single-item measures indicates improvement.
‡ P values are from a mixed model, with baseline scores as the covariate, treatment and time as fixed effects, and the patient identifier as a random effect.

travascular hemolysis and the scavenging of nitric oxide by cell-free hemoglobin.[9-11] In this study, the reduction in intravascular hemolysis with eculizumab, as compared with placebo, was associated with a significant improvement in fatigue, as assessed by scores on the FACIT-Fatigue instrument. Eculizumab increased the baseline score for fatigue by 6.4 points. A change of three or more points in scores on this instrument represents a clinically important difference.[20] Improvement with eculizumab in the fatigue component of the EORTC QLQ-C30 instrument provides additional evidence for the benefit shown by scores on the FACIT-Fatigue instrument. These improvements with eculizumab occurred without complete resolution of the anemia, providing further evidence of the contribution of hemolysis, in contrast to anemia, to the diminishing quality of life of patients with PNH. Clinical assessment of additional symptoms related to the quality of life of such patients, including abdominal pain, dysphagia, and erectile dysfunction, have also been reported to improve during eculizumab therapy.[21]

There were no deaths during the study, and only a single thrombotic event occurred in a patient in the placebo group. There were four serious adverse events in the eculizumab group and nine in the placebo group; all these patients recovered. The issue of possible protection against the risk of thrombosis through terminal complement inhibition with eculizumab is being evaluated in ongoing clinical studies of PNH. All 85 patients who completed the study elected to receive eculizumab in an open-label extension study.

The results of this randomized, double-blind, controlled study show that terminal complement inhibition with eculizumab reduces intravascular

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Table 4. Adverse Events.*

Adverse Event	Placebo Group (N=44)	Eculizumab Group (N=43)
	no. (%)	
Total no. of serious adverse events	9 (20)	4 (9)
Exacerbation of PNH	3 (7)	1 (2)
Renal colic	0	1 (2)
Lumbar- or sacral-disk prolapse	0	1 (2)
α-Hemolytic streptococcal bacteremia	0	1 (2)
Central-line and urinary tract infections	1 (2)	0
Upper respiratory tract infection	1 (2)	0
Probable viral infection	1 (2)	0
Neutropenia	1 (2)	0
Cellulitis, folliculitis, and neutropenia	1 (2)	0
Anemia and pyrexia	1 (2)	0
Most frequent adverse events†		
Headache‡	12 (27)	19 (44)
Nasopharyngitis	8 (18)	10 (23)
Upper respiratory tract infection	10 (23)	6 (14)
Back pain	4 (9)	8 (19)
Nausea	5 (11)	7 (16)
Cough	4 (9)	5 (12)
Diarrhea	5 (11)	4 (9)
Arthralgia	5 (11)	3 (7)
Abdominal pain	5 (11)	2 (5)
Dizziness	5 (11)	2 (5)
Vomiting	5 (11)	2 (5)
Fatigue	1 (2)	5 (12)
Viral infection	5 (11)	1 (2)

* Adverse events were coded with the use of preferred terms from the MedDRA.
† The event occurred in at least 10% of patients in either group.
‡ After the first 2 weeks of treatment, 10 patients (23%) receiving placebo and 9 patients (21%) receiving eculizumab had headache.

hemolysis, reduces or eliminates the need for transfusion, and improves anemia, fatigue, and the quality of life in patients with PNH. The data provide support for the central role of intravascular hemolysis in the pathogenesis of the disease and indicate that eculizumab is an effective treatment in patients with PNH.

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APPENDIX

In addition to the authors, the following investigators and institutions participated in the TRIUMPH study: **Australia** — Princess Alexandra Hospital, Woolloongabba: A. Mills; Queen Elizabeth Hospital, Woodville South: J. Norman; Royal Melbourne Hospital, Parkville; Royal Perth Hospital, Perth, WA: R. Herrmann; **Belgium** — St. Luc University Hospital, UCL, Brussels: E. Van Den Neste; **Canada** — University of Alberta, Cross Cancer Institute, Edmonton, AB.: L. Larratt, A. Turner, M.A. Hamilton; **Germany** — Universitätsklinikum Essen, Essen: U. Dührsen; Medizinische Hochschule Hanover, Hanover: A. Ganser; Universitätsklinik Greifswald, Greifswald: M. Montemurro; Institut für Klinische Transfusionsmedizin and Immungenetik, University Hospital Ulm, Ulm; Saarland University Medical School, Hamburg; **France** — Hospital de l'Hotel-Dieu, Paris: B. Rio; Hospital St. Louis and INSERM, Paris; **Ireland** — St. James Hospital, Dublin; **Italy** — Ospedale San Martino, Genoa: A. Bacigalupo; Azienda-Ospedaliera Universitaria Careggi, Florence: E. Antonioli, G. Gianfaldoni, F. Mannelli, A. Bosi; Ospedale San Bortolo, Vicenza: F. Rodeghiero; Federico II University, Naples: B. Rotoli, F. Alfinito; Ospedale Maggiore di Milano, Milan: A. Zanella, C. Boschetti; Istituto Toscana Tumori, Florence; **the Netherlands** — Radboud University Medical Center, Nijmegen; **Sweden** — Lund University Hospital, Lund: P.-G. Nillson; Umea University Hospital, Umea: A. Wahlin; Stockholm South Hospital, Stockholm: J. Samuelsson, L.G. Lundberg, P. Andersson; **United Kingdom** — St. George's Hospital, London; Leeds General Infirmary, Leeds; Belfast City Hospital, Belfast: M.F. McMullin; **United States** — Washington University School of Medicine, St. Louis: M. Bessler, L. Andritsos, M. Blinder, S. Devine; Johns Hopkins University Medical Center, Baltimore; Memorial Sloan-Kettering Cancer Center, New York: H. Castro-Malaspina, D. Araten; Stanford University Medical Center, Stanford, CA: S. Coutre; Duke University Medical Center, Durham, NC: C. de Castro III; Cleveland Clinic Florida, Weston, FL: E. Stone; University of Pennsylvania, Philadelphia: B. Konkle; Massachusetts General Hospital, Boston: D. Kuter; Cleveland Clinic Foundation, Cleveland: A. Lichtin; New York University Clinical Cancer Center, New York: T. Moskovits, B.G. Raphael, E. Amorosi, K.B. Hymes, P. Cook; City of Hope National Medical Center, Duarte, CA; Indiana University Cancer Center, Indianapolis: R. Nelson; University of California at Los Angeles, Los Angeles: R. Paquette; Hartford Hospital, Hartford, CT: R. Siegel; National Heart, Lung, and Blood Institute, Bethesda, MD: B. Savani.

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MINIREVIEW

Antibody Structure, Instability, and Formulation

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ABSTRACT: The number of therapeutic monoclonal antibody in development has increased tremendously over the last several years and this trend continues. At present there are more than 23 approved antibodies on the US market and an estimated 200 or more are in development. Although antibodies share certain structural similarities, development of commercially viable antibody pharmaceuticals has not been straightforward because of their unique and somewhat unpredictable solution behavior. This article reviews the structure and function of antibodies and the mechanisms of physical and chemical instabilities. Various aspects of formulation development have been examined to identify the critical attributes for the stabilization of antibodies. © 2006 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 96:1–26, 2007

Keywords: biotechnology; stabilization; protein formulation; protein aggregation; freeze drying/lyophilization

INTRODUCTION

Protein therapies are entering a new era with the influx of a significant number of antibody pharmaceuticals. Generally, protein drugs are effective at low concentrations with less side effects relative to small molecule drugs, even though, in rare cases, protein-induced antibody formation could be serious.[1] Therefore, this category of therapeutics is gaining tremendous momentum and widespread recognition both in small and large drug firms. Among protein drug therapies, antibodies play a major role in controlling many types of diseases such as cancer, infectious diseases, allergy, autoimmune diseases, and inflammation. Since the approval of the first monoclonal antibody (MAb) product -OKT-3 in 1986, more than 23 MAb drug products have entered the market (Tab. 1). The estimated number of antibodies and antibody derivatives constitute 20% of biopharmaceutical products currently in development (about 200).[2] The global therapeutic antibody market was predicted to reach $16.7 billion in 2008.[3]

There are several reasons for the increasing popularity of antibodies for commercial development. First, their action is specific, generally leading to fewer side effects. Second, antibodies may be conjugated to another therapeutic entity for efficient delivery of this entity to a target site, thus reducing potential side effects. For instance, Mylotarg is an approved chemotherapy agent composed of calicheamicin conjugated to humanized IgG4, which binds specifically to CD33 for the treatment of CD33-positive acute myeloid leukemia. Another example is the conjugation of immunotoxic barnase with the light chain of the anti-human ferritin monoclonal antibody F11 as potential targeting agents for cancer immunotherapy.[4] Third, antibodies may be conjugated to radioisotopes for specific diagnostic purposes. Examples include CEA-Scan for detection of colorectal cancer and ProstaScint for detection of prostate cancer. Lastly, technology advancement has made complete human MAb available, which are less immunogenic.

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Table 1. Commercial Monoclonal Antibody Products

#	Brand name	Molecule	MAb	Year	Company	Route	Indication	MAb Conc	Buffer	Excipients	Surfactant	pH
1	Avastin	Bevacizumab	Humanized IgG1, 149 kDa	2004	Genetech and BioOncology	IV infusion	Metastatic carcinoma of colon or rectum, binds VEGF	100 mg and 400 mg/vial (25 mg/mL) solution	5.8 mg/mL monobasic NaPhos H_2O; 1.2 mg/mL dibasic NaPhos anhydrous (4 mL, 16 mL fill in vial)	60 mg/mL α-Trehalose dihydrate (4 mL, 16 mL fill in vial)	0.4 mg/mL PS20 (4 mL, 16 mL fill in vial)	6.2
2	Bexxar	Tositumomab and I-131Tositumab	Murine IgG2λ	2003	Corixa and GSK	IV Infusion	CD20 positive follicular nonHodgkins lymphoma	Kit: 14 mg/mL MAb solution in 35 mg and 225 mg vials; 1.1 mg/mL *I131-MAb* solution	10 mM phosphate (MAb vial)	145 mM NaCl, 10 % w/v Maltose; *I131-MAb:* 5–6% Povidone, 1–2, 9–15 mg/mL Maltose, 0.9 mg/mL NaCl, 0.9–1.3 mg/mL Ascorbic acid		7.2
3	Campath	Alemtuzumab	Humanized, IgG1k, 150 kDa	2001	Ilex Oncology; Millenium and Berlex	IV infusion	B-cell chronic lymphocytic leukemia, CD52-antigen	30 mg/3 mL solution	3.5 mg/3 mL dibasic NaPhos, 0.6 mg/3 mL monobasic KPhos	24 mg/3 mL NaCl, 0.6 mg/3 mL KCl, 0.056 mg/3 mL Na2EDTA	0.3 mg/3 mL PS80	6.8–7.4
4	CEA-Scan (lyo)	Arcituomab; Tc-99	Murine Fab, 50 kDa	1996	Immunomedics	IV injection or infusion	Imaging agent for colorectal cancer	1.25 mg/vial Lyophilized MAb. Reconstitute w 1 mL Saline w Tc99m	0.29 mg/vial Stannous chloride, potassium sodium tartrate tetrahydrate, NaAcetate.$3H_2O$, NaCl, glacial acetic acid, HCl	Sucrose		5.7
5	Erbitux	Cetuximab	Chimeric human/ mouse IgG1k, 152 kDa	2004	ImClone and BMS	IV infusion	Treatment of EGFR-expressing colorectal carcinoma	100 mg MAb in 50 mL; 2 mg/ mL solution	1.88 mg/mL DibasicNaPhos · $7H_2O$; 0.42 mg/mL MonobasicNaPhos · H_2O	8.48 mg/mL NaCl		7.0–7.4
6	Herceptin (lyo)	Trastuzumab	Humanized IgG1k	1998	Genetech	IV infusion	Metastatic breast cancer whose tumor overexpress HER2 protein	440 mg /vial, 21 mg/mL after reconstitution	9.9 mg/20 mL L-HistidineHCl, 6.4 mg/20 mL L-Histidine	400 mg/20 mL α-Trehalose Dihydrate	1.8 mg/20 mL PS20	6
7	Humira	Adalimumab	Human IgG1k, 148 kDa	2002	CAT and Abbott	SC	RA patients not responding to DMARDs. Blocks TNF-alpha	40 mg/0.8 mL solution (50 mg/mL)	0.69 mg/0.8 mL Monobasic NaPhos · $2H_2O$; 1.22 mg/0.8 mL DibasicNaPhos · $2H_2O$; 0.24 mg/0.8 mL NaCitrate, 1.04 mg/ 0.8 mL Citric acid · H_2O	4.93 mg/0.8 mL NaCl; 9.6 mg/0.8 mL Mannnitol	0.8 mg/0.8 mL PS80	5.2
8	Lucentis	Ranibizumab	Humanized IgG1k fragment	2006	Genentech	Intravitreal injection	Age-related macular degeneration (wet)	10 mg/mL solution	10 mM HistidineHCl	10% α-Trehalose-Dihydrate	0.01% PS20	5.5

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9	Mylotarg (lyo)	Gemtuzumab ozogamicin	Humanized IgG4k conjugated with calicheamicin	2000	Celltech and Wyeth	IV infusion	Humanized Ab linked to calicheamicin for treatment of CD33 positive acute myeloid leukemia	5 mg protein-equivalent lyophilized powder/20-mL vial	Monobasic and dibasic NaPhosphate	Dextran 40, Sucrose, NaCl		
10	OncoScint	Satumomab pendetide	Murine IgG1k conjugated to GYK-DTPA	1992	Cytogen	IV injection	Imaging agent for colorectal and ovarian cancer	0.5 mg conjugate/mL solution (2 mL per vial)	Phosphate buffer saline			6.0
11	Orthoclone OKT	Muromomab-CD3	Murine, IgG2a, 170 kDa	1986	Ortho Biotech	IV injection	Reversal of acute kidney transplant rejection (anti CD3-antigen)	1 mg/mL solution	2.25 mg/5 mL monobasic NaPhos, 9.0 mg/5 mL dibasic NaPhos	43 mg/5 mL NaCl	1 mg/mL PS80	7 ± 0.5
12	ProstaScint	Indium-111 capromab pendetide	Murine IgG1k-conjugated to GYK-DTPA	1996	Cytogen	IV injection	Imaging agent for prostate cancer	0.5 mg conjugate/mL solution (1 mL per vial)	Phosphate buffer saline			5–7
13	Raptiva (lyo)	Efalizumab	Humanized IgG1k	2003	Xoma and Genentech	SC	Chronic moderate to severe plaque psoriasis, binds to CD11a subunit of LFA-1	150 mg MAb/vial; 125 mg/ 1.25 mL (100 mg/mL) after reconstitution with 1.3 mL SWFI	6.8 mg/vial L-HistidineHCl · H_2O; 4.3 mg/vial L-Histidine	123.2 mg/vial Sucrose	3 mg/vial PS20	6.2
14	Remicade (lyo)	Infliximab	Chimeric human/ murine MAb against TNFalpha (app. 30% murine, 70% corresponds to human IgG1 heavy chain and human kappa light chain constant regions)	1998	Centocor	IV infusion	RA and Crohn's disease (anti TNFalpha)	100 mg / 20-mL Vial, 10 mg/mL on reconstitution	2.2 mg/10 mL MonobasicNaPhos H_2O, 6.1 mg/10 mL DibasicNaPhos · $2H_2O$	500 mg/10 mL Sucrose	0.5 mg/10 mL PS80	7.2
15	ReoPro	Abciximab	Fab, Chimeric human-murine, 48 kDa	1994	Centocor / Lilly	IV injection and infusion	Reduction of acute blood clot related complications	2 mg/mL solution	0.01 M NaPhosphate	0.15 M NaCl	0.001% (0.01 mg/ mL) PS80	7.2
16	Rituxan	Rituximab	Chimeric mouse/ human IgG1k with murine light and heavy chain variable region (Fab domain), 145 kDa	1997	IDEC and Genentech	IV infusion	NonHodgkin's lymphoma. (anti CD20-antigen)	10 mg/mL solution	7.35 mg/mL NaCitrate · $2H_2O$	9 mg/mL NaCl	0.7 mg/mL PS80	6.5

(*Continued*)

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Table 1. (*Continued*)

#	Brand name	Molecule	MAb	Year	Company	Route	Indication	MAb Conc	Buffer	Excipients	Surfactant	pH
17	Simulect (lyo)	Basiliximab	Chimaric IgG1k, 144 kDa	1998	Novartis	IV injection and infusion	Prevention of acute kidney transplant rejection, IL-2 receptor antagonist	10 mg and 20 mg/ vial, 4 mg/mL on reconstitution	3.61 mg, 7.21 mg Monobasic KPhos; 0.50 mg, 0.99 mg Na_2HPO_4	0.8 mg, 1.61 mg NaCl; 10 mg, 20 mg Sucrose; 40 mg, 80 mg Mannitol; 20 mg 40 mg Glycine		
18	Synagis (lyo)	Palivizumab	Humanized IgG1k, CDR of murine MAb 1129, 148 kDa	1998	MedImmune	IM injection	Prevent replication of the Respiratory syncytial virus (RSV)	50 mg and 100 mg / vial, 100 mg/mL on reconstitution	47 mM Histidine, 3.0 mM Glycine	5.6 % Mannitol		
19	Tysabri	Natalizumab	Humainzed IgG4k	2004	Biogen IDEC	IV Infusion	MS relapse	300 mg/15 mL solution	17.0 mg MonobasicNa Phos·H_2O, 7.24 mg diBasicNaPhos·$7H_2O$ for 15 mL	123 mg/15 mL NaCl	3.0 mg/15 mL PS80	6.1
20	Verluma	Nofetumomab	Murine Fab	1996	Boehringer Ingelheim and DuPont Merck	IV injection	Imaging agent for lung cancer	10 mg/mL solution	Phosphate buffer saline			?
21	Xolair (lyo)	Omalizumab	Humanized IgG1k, 149 kDa		Genentech w Novartis and Tanox	SC	Asthma, inhibits binding of IgE to IgE receptor FCeRI	202.5 mg/vial, Deliver 150 mg/ 1.2 mL on reconstitution with 1.4 mL SWFI	2.8 mg LHistidineHCl·H_2O; 1.8 mg LHistidine	145.5 mg Sucrose	0.5 mg PS20	
22	Zenapax	Daclizumab	Humanized IgG1, 144 kDa	1997	Roche	IV infusion	Prophylaxis of acute organ rejection in patients receiving renal transplants. Inhibits IL-2 binding to the Tac subunit of IL-2 receptor complex	25 mg/5 mL MAb Solution	3.6 mg/mL MonobasicNa Phos·H_2O; 11 mg/mL DibasicNaPhos·$7H_2O$	4.6 mg/mL NaCl	0.2 mg/mL PS80	6.9
23	Zevalin	Ibritumomab-Tiuxetan	Murine IgG1k-thiourea covalent linkage to Tiuxetan		IDEC	IV infusion	CD20 antigen. (Kit with Ytterium-90 induces cellular damage by beta emission)	3.2 mg/2 mL solution		09% NaCl		7.1

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Development of commercially viable antibody pharmaceuticals has, however, not been straightforward. This is because the behavior of antibodies seems to vary, even though they have similar structures. In attempting to address some of the challenges in developing antibody therapeutics, Harris et al.[5] reviewed the commercial-scale formulation and characterization of therapeutic recombinant antibodies. In a different review, antibody production and purification have been discussed.[2] Nevertheless, the overall instability and stabilization of antibody drug candidates have not been carefully examined in the literature. This article, not meant to be exhaustive, intends to review the structure and functions of antibodies, discuss their instabilities, and summarize the methods for stabilizing/formulating antibodies.

ANTIBODY STRUCTURE

Antibodies (immunoglobulins) are roughly Y-shaped molecules or combination of such molecules (Fig. 1). Their structures are divided into two regions—the variable (V) region (top of the Y) defining antigen-binding properties and the constant (C) region (stem of the Y), interacting with effector cells and molecules. Immunoglobulins can be divided into five different classes —IgA, IgD, IgE, IgM, and IgG based on their C regions, respectively designated as α, δ, ε, μ, and γ (five main heavy-chain classes).[6] Most IgGs are monomers, but IgA and IgM are respectively, dimmers and pentamers linked by J chains. IgGs are the most abundant, widely used for therapeutic purposes, and their structures will be discussed as antibody examples in detail.

Primary Structure

The structure of IgGs have been thoroughly reviewed.[6] The features of the primary structure of antibodies include heavy and light chains, glycosylation, disulfide bond, and heterogeneity.

Heavy and Light Chains

IgGs contain two identical heavy (H, 50 kDa) and two identical light (L, 25 kDa) chains (Fig. 1). Therefore, the total molecular weight is approximately 150 kDa. There are several disulfide bonds linking the two heavy chains, linking the heavy and light chains, and residing inside the chains (also see next section). IgGs are further divided

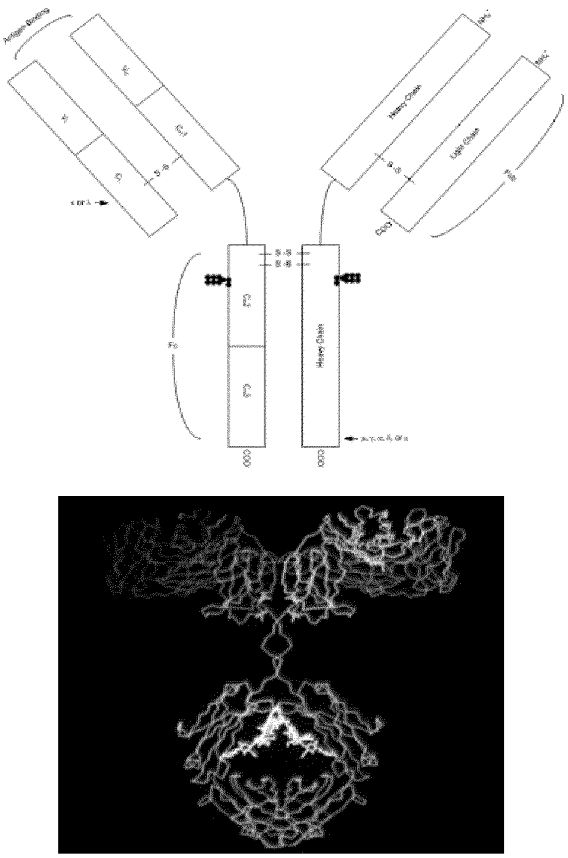


Figure 1. Linear (upper panel) and steric (lower panel) structures of immunoglobulins (IgG).

into several subclasses—IgG1, IgG2, IgG3, and IgG4 (in order of relative abundance in human plasma), with different heavy chains, named γ1, γ2, γ3, and γ4, respectively. The structural differences among these subtypes are the number and location of interchain disulfide bonds and the length of the hinge region. The light chains consist of two types—lambda (λ) and kappa (κ). In mice, the average of κ to λ ratio is 20:1, whereas it is 2:1 in humans.[6] The variable (V) regions of both chains cover approximately the first 110 amino acids, forming the antigen-binding (Fab) regions, whereas the remaining sequences are constant (C) regions, forming Fc (fragment crystallizable) regions for effector recognition and binding.[6] The N-terminal sequences of both the heavy and light chains vary greatly between different antibodies. It was suggested that the conserved sequences in human IgG1 antibodies

are approximately 95% and the remaining 5% is variable and creates their antigen-binding specificity.[5]

The V regions are further divided into three hypervariable sequences (HV1, HV2, and HV3) on both H and L chains. In the light chains, these are roughly from residues 28 to 35, from 49 to 59, and from 92 to 103, respectively.[6] Other regions are the framework regions (FR1, FR2, FR3, and FR4). The HV regions are also called the complementarity determining regions (CDR1, CDR2, and CDR3). While the framework regions form the β-sheets, the HV sequences form three loops at the outer edge of the β barrel (also see Section 2.2).

Disulfide Bonds

Most IgGs have four interchain disulfide bonds—two connecting the two H chains at the hinge region and the other two connecting the two L chains to the H chains.[6] Exceptions do exist. Two disulfide bonds were found in IgG1 and IgG4 linking the two heavy chain in the hinge region but four in IgG2.[7] In IgG1 MAb, HC is linked to the LC between the fifth Cys (C217) of HC and C213 on the LC. In IgG2 and IgG4 MAbs, it is the third Cys of HC (C123) linking to the LC.[7] A disulfide bond between HC C128 and LC C214 was found for mouse catalytic monoclonal antibodies (IgG2a).[8]

IgGs have four intrachain disulfide bonds, residing in each domain of the H and L chains, stabilizing these domains. The intrachain disulfide bonds in V_H and V_L are required in functional antigen binding.[9] Native IgG MAbs should not have any free sulfhydryl groups.[7] However, detailed examination of the free sulfhydryl groups in recombinant MAbs (one IgG1, two IgG2, and one IgG4) suggests presence of a small portion of free sulfhydryl group (approximately 0.02 mol per mole of IgG2 or IgG4 MAb and 0.03 for IgG1.[7] In rare cases, a free cysteine is found. A nondisulfide-bonded Cys at residue 105 was found on the heavy chain of a mouse monoclonal antibody, OKT3 (IgG2a).[10]

Oligosaccharides

There is one oligosaccharide chain in IgGs.[6] This N-linked biantennary sugar chain resides mostly on the conserved Asn 297, which is buried between the C_H2 domains.[5,11] For example, the oligosaccharide resides on Asn-297 of the C_H2 domain of chimeric IgG1 and IgG3 molecules[12] but on Asn 299 in a monoclonal antibody, OKT3 (IgG2a).[10] The oligosaccharide, often microheterogeneous, is typically fucosylated in antibodies produced in CHO or myeloma cell lines[5] and may differ in other cell lines.[2,11] There are many factors that dictate the nature of the glycan microheterogenity on IgGs. These include cell line, the bioreactor conditions and the nature of the downstream processing. An additional oligosaccharide can be found in rare cases. A human IgG produced by a human-human-mouse heterohybridoma contains an additional oligosaccharide on Asn 75 in the variable region of its heavy chain.[13] In addition, O-linked carbohydrates could also exist in this antibody.

Proper glycosylation is critical for correct functioning of antibodies.[11] It was demonstrated that removal of the oligosaccharide in IgGs (IgG1 and IgG3) made them ineffective in binding to C1q, in binding to the human FcγRI and activating C; and generally more sensitive to most proteases than their corresponding wild-type IgGs (one exception).[12] This is because the binding site on IgG for C1q, the first component of the complement cascade, is localized in the C_H2 domains.[11] Furthermore, the glycosylation can affect the antibody conformation.[12]

Oligosaccharides in other regions can also play a critical role. Removal of an oligosaccharide in a Fv region of the CBGA1 antibody resulted in a decreased antigen-binding activity in several ELISA systems.[13] In addition, this oligosaccharide might play critical role in reducing the antigenicity of the protein.[14]

The sugar composition of the oligosaccharide is also critical in antibody functions. It has been shown that a low fucose (Fuc) content in the complex-type oligosaccharide in a humanized chimeric IgG1 is responsible for a 50-fold higher antibody-dependent cellular cytotoxicity (ADCC) compared with a high Fuc counterpart.[15]

Heterogeneity

Purified antibodies are heterogeneous in structure. This is true for all monoclonal antibodies (MAbs) due to differences in glycosylation patterns, instability during production, and terminal processing.[5] For example, five charged isoforms were found in recombinant humanized monoclonal antibody HER2 as found by capillary isoelectric focusing (cIEF) and sodium dodecyl sulfate–capillary gel electrophoresis (SDS–CGE).[16] Six separate bands were focused under

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IEF for two mouse monoclonal antibodies IgG2a (κ) and IgG1 (κ).[17] A mature monoclonal antibody, OKT3 (IgG2a), contain cyclized N-terminus (pyroglutamic acid, −17 D) in both H and L chains, processed C-terminus (no Lys, −128 D) of the H chains, and a small amount of deamidated form.[10] Similar observation was also reported for a humanized IgG1 (κ).[18] In rare cases, gene cross-over may lead to formation of abnormal heavy chains. For example, a purified monoclonal anti-IgE antibody contains a small amount of a variant H chain, which had 16 fewer amino acid residues than the normal H chain (position is between Arg108 of the L chain and Ala124 of the H chain).[19]

Secondary and Higher-Order Structure

The basic secondary and higher-order structural features of IgGs have been reviewed.[6] Only a small portion of the three-dimensional structures of IgGs has been solved.[20] The antibody's secondary structure is formed as the polypeptide chains form anti-parallel β-sheets. The major type of secondary structure in IgGs is these β-sheets and its content is roughly 70% as measured by FTIR.[21] The light chain consists of two and the heavy chain contains four domains, each about 110 amino acid long.[6,20] All these domains have similar folded structures—β barrel, also called immunoglobulin fold, which is stabilized by a disulfide bond and hydrophobic interaction (primary). These individual domains (~12 kDa in size) interact with one another (V_H and V_L; C_H1 and C_L; and between two C_H3 domains except the carbohydrate-containing C_H2 domain) and fold into three equal-sized spherical shape linked by a flexible hinge region. These three spheres form a Y shape (mostly) and/or a T shape.[22]

The less globular shape of IgGs is maintained both by disulfide bonds and by strong noncovalent interactions between the two heavy chains and between each of the heavy-chain/light-chain pairs.[23] Through noncovalent interactions, a less stable domain becomes more stable, and thus, the whole molecule can be stabilized.[24] A detailed study indicates that the interaction between two CH3 domains are dominated by six contact residues, five of these residues (T366, L368, F405, Y407, and K409) forming a patch at the center of the interface.[25] These noncovalent interactions are spatially oriented such that variable domain exchange (switching V_H and V_L; inside-out IgG; ioIgG) induces noncovalent multimerization.[26]

The six hypervariable regions in CDR (L1, L2, L3, H1, H2, and H3) form loops of a few predictable main-chain conformations (or canonical forms), except H3 loop, which has too many variations in conformation to be predicted accurately.[27,28] There is a slight difference in the loop composition and shape between the two types of light chains.[20] However, no functional difference was found in antibodies having λ or κ chain.[6]

Basic Functions of Antibodies

The basic functions of antibodies have been reviewed.[6] There are two functional areas in IgGs—the V and C regions. The V regions of the two heavy and light chains offer two identical antigen-binding sites. The binding of the two sites (bivalent) can be independent of each other and does not seem to depend on the C region.[29] The exact antigen-binding sites are the CDR regions with participation of the frame work regions.[30] Binding of antigens seems through the induced-fit mechanism.[31,32] The induced-fit mechanism allows multispecificity and polyreactivity. It has been suggested that about 5–10 residues usually contribute significantly to the binding energy.[32]

The C regions of antibodies have three main effector functions (1) being recognized by receptors on immune effector cells, initiating antibody-dependent cell cytotoxicities (ADCC), (2) binding to complement, helping to recruit activated phagocytes, and (3) being transported to a variety of places, such as tears and milk.[6] In addition, C domains also modulate *in vivo* stability.[23,29,33] The function of Fc is affected by the structure of Fab. Variable domain exchange (switching V_H and V_L; inside-out IgG; ioIgG) affected Fc-associated functions such as serum half-life and binding to protein G and FcγRI.[26]

The hinge region provides flexibility in bivalent antigen binding and activation of Fc effector functions.[26] Two chimeric IgG3 antibodies lacking a genetic hinge but with Cys residues in CH2 regions was found to be deficient in their intermolecular assembly, and both IgG3 ΔH + Cys and IgG3 ΔH + 2Cys lost greatly their ability to bind FcγRI and failed to bind C1q and activate the complement cascade.[34]

Alternative Forms of Antibodies

In addition to species-specific antibodies, other antibody forms are generated to meet various needs. In the early development of antibody therapies, antibodies were made from murine

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sources. However, these antibodies easily elicit formation of human anti-mouse antibody (HAMA). Therefore, humanized chimeric antibodies were generated. Chimeric monoclonal antibodies (60–70% human) are made of mouse variable regions and human constant regions.[2] Such antibodies can still induce formation of human anti-chimeric antibody (HACA). Highly humanized antibodies, CDR-grafted antibodies, are made by replacing only the human CDR with mouse CDR regions (90–95% human).[2] These antibodies are almost the same in immunogenicity potential as completely human antibodies, which may illicit formation of human anti-human antibody (HAHA).

Other alternative forms of antibodies have also been generated and these different forms have been reviewed.[35] Treatment with papain would cleave the N-terminal side of the disulfide bonds and generate two identical Fab fragments and one Fc fragment. Fab's are 50 kDa ($V_H + C_H1$)/($V_L + C_L$) heterodimers linked by a single disulfide bond. Treatment with pepsin cleaves the C-terminal side of the disulfide bonds and produces a $F(ab)'_2$ fragment. The remaining H chains were cut into several small fragments.[6] Cleavage by papain occurs at the C-terminal side of His-H228[36] or His-H227.[37] Reduction of $F(ab')_2$ will produce two Fab'.[23]

Fv fragments are noncovalent heterodimers of V_H and V_L. Stabilization of the fragment by a hydrophilic flexible peptide linker generates single-chain Fv (scFvs).[2] Fragments without constant domains can also be made into domain antibodies (dAbs). These scFvs are 25–30 kDa variable domain ($V_H + V_L$) dimers joined by polypeptide linkers of at least 12 residues. Shorter linkers (5–10 residues) do not allow pairing of the variable domains but allow association with another scFv form a bivalent dimer (diabody) (about 60 kDa, or trimer: triabody about 90 kDa).[38] Two diabodies can be further linked together to generate bispecific tandem diabody (tandab).[39] Disulfide-free scFv molecules are relatively stable and useful for intracellular applications of antibodies—"intrabodies."[38] The smallest of the antibody fragments is the minimal recognition unit (MRU) that can be derived from the peptide sequences of a single CDR.[2]

ANTIBODY INSTABILITY

Antibodies, like other proteins, are prone to a variety of physical and chemical degradation pathways, although antibodies, on the average, seem to be more stable than other proteins. Antibody instabilities can be observed in liquid, frozen, and lyophilized states. The glycosylation state of an antibody can significantly affect its degradation rate.[40] In many cases, multiple degradation pathways can occur at the same time and the degradation mechanism may change depending on the stress conditions.[41] These degradation pathways are divided into two major categories—physical and chemical instabilities. This section will explore the possible degradation pathways of antibodies and their influencing factors.

Physical Instability

Antibodies can show physical instability via two major pathways—denaturation and aggregation.

Denaturation

Antibodies can denature under a variety of conditions. These conditions include temperature change, shear, and various processing steps. Compared with other proteins, antibodies seem to be more resistant to thermal stress. They may not melt completely until temperature is raised above 70°C,[21,42,43] while most other mesophilic proteins seem to melt below 70°C.[44] Shear may cause antibody denaturation. For example, the antigen-binding activity of a recombinant scFv antibody fragment was reduced with a first-order rate constant of 0.83/h in a buffer solution at a shear of approximately 20,000/s.[45]

Lyophilization can denature a protein to various extents. An anti-idiotypic antibody (MMA 383) in a formulation containing mannitol, saccharose, NaCl, and phosphate was found to loose its *in vivo* immunogenic properties (only 10–20% of normal response rate) upon lyophilization.[46] Since the protein showed no evidence of degradation after lyophilization, no change in secondary structure by CD (29% β-sheet, 14% α-helix, and 57% "other"), the loss of activity was attributed to the conformational change. Indeed, tryptophan fluorescence properties were different between the lyophilized and unlyophilized antibodies.[46]

Aggregation

Antibody aggregation is a more common manifestation of physical instability. The concentration-dependent antibody aggregation was considered the greatest challenge to developing protein formulations at higher concentrations.[47] This is

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because protein aggregates generally have reduced activity and more importantly, greater immunogenicity potential because of the multiplicity of epitopes and/or conformational changes.[14,48] Immunoglobulin aggregates have been shown to cause serious renal failure[49] and anaphylactoid reactions such as headache, fever, and chills.[50] Therefore, the aggregate level in commercial intravenous immunoglobulin products is limited to less than 5% based on the WHO standards.

Aggregates can form easily both in liquid and solid states under a variety of conditions (Tab. 2). Since protein aggregation is often the consequence of protein–protein interactions, a process influenced by diffusion rate and geometric constraints of the interaction sites, such factors would influence significantly the aggregation rate, including protein concentration change, viscosity, ionic strength, pH, and temperature.[51] Other conditions may also affect protein aggregation, including shaking, long-term storage, freeze-thaw process, lyophilization process, etc.

Increasing the concentration of antibodies often increases the aggregation tendency of the protein. It was demonstrated that increasing the IgG1 concentration (in 10 mM citric acid, 100 mM NaCl pH 5.5) from 2.7 mg/mL to 50 mg/mL almost linearly increased the Nephelometric Unit (NU) from 2 to 40.[52] Since EP defines a clear solution as having equal or less than three NU, most antibody solutions in the study are opalescent except those at 5 mg/mL or less. However, as the weight average molecular weight was found to be 0.9–1.3 times that expected of a monomer (about 149 kDa), minimal protein–protein association (and readily reversible) is suggested.[52] Shaking can accelerate antibody precipitation and the shape of the precipitates may depend on the sample volume in a container.[53] It appears that protein solutions exhibiting lower surface tension are more susceptible to protein denaturation and precipitation.[53]

Low-temperature treatment may induce aggregation of antibodies. The reversible low-temperature-induced aggregation (below 37°C) of serum cryoglobulins is well known.[54] Human IgM cryoglobulin preparations easily precipitate or gel at temperatures below 10–12°C and the process is reversible at a higher temperature.[20] In a recent study, aggregation of IgG1 at low temperature at above 18 mg/mL was reversible as measured by light scattering.[52] The low-temperature-induced aggregation of cryoglobulins is poorly understood and was thought to involve sites within both Fab and Fc.[20] The authors of this article believe that low temperature reduces the hydrophobic interaction, which is the major force in protein folding. Without enough hydrophobic interaction at low temperature, hydrophobic regions of antibodies become more exposed to solvent and lead to increased intermolecular hydrophobic interaction, leading to aggregation.

In close relation to the low-temperature effect, freeze-thaw process often induces protein aggregation. However, freeze-thaw-induced antibody aggregation does not seem to be a major issue, partly due to the reversibility of antibody aggregates. Freeze-thawing of a chimeric (L6) antibody solution at pH 7.2 for as many as 35 times led to formation of dimers (not larger aggregates).[41] Maximum amount of dimers (about 20%) was observed at pH 6.5 and minimum was below 5.5 or above 8.5 (less than 2%). The freeze-thaw-induced aggregation is apparently reversible after treatment at 37°C for a few hours.[41] Freeze-thawing of rhuMAb anti-CD20 three times (freezing to either −20 or −70°C, thawing to 5°C) did not lead to significant change in aggregation, no significant loss of monomer by RP-HPLC or SEC-HPLC.[55] On the other hand, isolated Fabs seems to be more prone to freeze-thaw-induced aggregation relative to full-length antibodies. For example, Lee[56] found that freeze-thawing of a scF_v (MW 27,000 d) at 1.45 mg/mL in sodium phosphate buffered saline (PBS) at pH 7.3 dropped the %monomer content (due to aggregation) from 95% to 84%, 59%, 44%, and 37%, respectively after 1–4 cycles.

Lyophilization may induce antibody aggregation to a variable degree. It was demonstrated that lyophilization of a recombinant humanized monoclonal antibody (rhuMAb HER2), in the absence of sugars, led to a slight increase in aggregate (less than 1.2%).[57] Lyophilization caused detectable aggregation of a human monoclonal antibody in PBS or physiological saline as measured by OD600,[58] and aggregation of a mouse monoclonal antibody (MN12) [IgG2a (κ)] in the absence or presence of 5% sucrose, dextran, or hydroxypropyl-beta-cyclodextrin (HP-β-CD), as measured by visual examination.[59] In a different study, lyophilization of a monoclonal antibody-vinca conjugate in PBS at pH 7.4 caused 6.2% aggregation.[60] The lyophilization-induced aggregates could be non-covalent-linked monomers and/or even disulfide-linked.[61,62]

Antibody aggregation can easily occur during storage in a liquid state. Storing of a fully human anti-IL8 monoclonal antibody (ABX-IL8; IgG2)

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Table 2. Instabilities of Antibodies

Instability	Antibody	Formulation	Condition	Effect	References
Denaturation	scFv	3 μg/mL in 10 mM phosphate-buffered saline containing 0.02% sodium azide	Shaking at a mean velocity gradient approximately 20,000/s	Loss of activity at a first-order rate of 0.83/h	45
Aggregation	Chimeric mouse/ human IgG1	2 mg/mL in PBS at pH 7.2	Stirring for 48 h with Teflon bar at 600 RPM	Increased turbidity Formation of >15 k particles (>10 μ)	91
	Human Anti-IL-8 (IgG2)	55 mg/mL, 5 mM His, pH 6	Freeze/thaw three times (room/−70°C)	About 0.6% aggregates (by SEC-HPLC)	21
	Anti-IL-8 (IgG2)	100 mg/mL, 200 mM Sucrose, 15 mM Arg, 15 mM His, pH 6	Incubation at 40°C for 6 months	About 11% aggregates (by SEC-HPLC)	21
	rhuMAbE25	10 mg/mL in excipient-free solution	Spray drying Tin = 105°C, Tout = 50–55°C	5–6% aggregate (by SEC-HPLC)	65
	MAb (type not specified)	MAb at 40 mg/mL, 85 mM sucrose, 5 mM His at pH 6, 0.01% Tween 20	Lyophilization	Cloudy (OD350 > 0.2)	1
	Human Anti-IL-8 (IgG2)	50 mg/mL, 20 mM Gly, 4 mM His, 0.2% mannitol, 16 mM Glu, 0.03% Tween 20 pH 5.7	Incubation of lyophilized form at 37°C for 1 month	4.8% aggregates (by SEC-HPLC)	21
	RhuMAb HER2	50 mg/mL in 100 mM trehalose, 5 mM His at pH 6.0	Incubation of lyophilized form at 40°C for 23 months	9.7% aggregates (by SEC-HPLC)	57
Deamidation	OKT3 (IgG2a)	1 mg/mL, PBS at pH 7.0 + Tween80	Incubation at 37°C for 2 months	90% deamidation of Asn386	10
	LA298 (IgG1)	0.5 mg/mL in 10 mM citrate at pH 7	Incubation at 25°C for 6 weeks	~12% increase in deamidation at Asn55	78
Isomerization	MAb	Lyophilized at 40 mg/mL, 85 mM sucrose, 5 mM His, 0.01% Tween 20, pH 6.0, containing 8.4% moisture	Incubation at 5°C	First-order rate constant $\sim 0.33 \times 10^{-4}$/day	1
Oxidation	rhuMAb HER2	5 mg/mL, 147 mM NaCl, 0.01% polysorbate 20, 5 mM sodium acetate at pH 5.0	5, 30, and 40°C for 2 weeks	10, 17, and 52% (total peak area)	81

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in aqueous solutions at 2–8°C or 25°C led to formation of high-molecular-weight (HMW) species.[21] Incubation of two mouse monoclonal antibodies IgG2a (κ) and IgG1 (κ) at 37°C for 32 days caused precipitation in pH 3 and 4 solutions for both antibodies.[17] Aggregation can also occur to an antibody conjugate. Storing a monoclonal antibody-vinca alkaloid conjugate (murine IgG2a) in phosphate-buffered saline solutions at 5°C between pH 4.5 and 7.4 for 1.5 months caused precipitation regardless of solution pH and aggregation was accelerated at 30°C throughout the pH range.[63]

Antibody aggregation is a common phenomenon during storage in a lyophilized state. Aggregation (OD350 > 0.2) was observed of a high concentration, lyophilized, monoclonal antibody (type not specified) by SEC under accelerated conditions.[1] So did a lyophilized chimeric monoclonal antibody during storage at 5°C (0.4% after 2 months).[64] In the absence of sugars, the extent of rhuMAb HER2 aggregation increased from about 1% to >10% after 3 months at 40°C and the rate of aggregation for these formulations was about 0.2% per month at 40°C.[57]

Antibody aggregation can readily occur in other solid forms during storage.[5] It was found that the primary degradation upon storage of a spray-dried anti-IgE (protein:mannitol = 80:20) is aggregation, and the amount of aggregates increased with increasing temperature and relative humidity (Maa et al., 1998a).[65] As much as 52% aggregate was observed by SEC-HPLC of a spray-dried anti-IgE (protein:mannitol = 80:20) after 1 year at 40°C and 38% RH (Maa et al., 1998b).[66]

Antibody aggregation is usually accelerated upon thermal treatment. In the study of chimeric (human/mouse) monoclonal antibody (BR96), Alexander and Hughes demonstrated that thermal treatment at 60°C for 166 h generated noncovalently linked aggregates.[67] In the evaluation of the relative stability of murine IgG2a (κ), chimeric IgG1(κ), trioma IgG1 (λ), and murine IgG1 (κ), Hartmann et al. demonstrated that IgG2a (κ) aggregated and precipitated after heating at 55°C for 8 h and the half-life for the loss of monomer for those four IgGs is 1, 18.1, 3.4, and 8.2 h with different aggregate species (dimer, trimer...) by SEC-HPLC.[68]

Surface Adsorption

Antibodies, like other proteins, can easily adsorb to a variety of surfaces. Surface adsorption can significantly reduce the antibody concentration in a solution. For example, a mouse monoclonal IgG1 has been shown to be adsorbed on the surface of glass shake flasks, causing a loss of protein in plant culture media.[69] Such a loss was minimized by coating the glass vessels or by addition of Pluronic F127.

Chemical Instability

A variety of chemical degradation pathways have been observed. These chemical degradants may or may not loose protein activity depending on the site of changes. For examples, deamidation of a monoclonal antibody, OKT3 (IgG2a) as measured by change in IEF pattern does not correlate with loss of antigen binding potency (90% deamidation of Asn386 vs. 80% activity in the potency assay).[10] In comparison, the activity of light chain deamidated recombinant humanized monoclonal antibody HER2 (rhuMAb HER2, Herceptin, trastuzuMAb) at Asn30 (to aspartate) is only 70% of the main species and that of the isomerized (heavy chain) asp102 species is only 9–21% of major species.[37] The major chemical degradation pathways in an antibody include cross-linking, deamidation, isomerization, oxidation, and fragmentation.

Disulfide Formation/Exchange

Disulfide bond formation/exchange is probably the most common cross-linking pathway, leading to chemical aggregation. This degradation pathway can occur readily in various processing steps. For example, lyophilization of a murine monoclonal antibody (IgG2a) without additives reduced the average number of –SH groups per molecule from 6.65 to 6.28, suggesting disulfide formation.[70] Since the thiolate ions are often the initiating species for this chemical degradation, increasing formulation pH usually leads to increased formation of disulfide formation. This is the case for an antifebrin T2G1s Fab′ (IgG1 (κ)), containing free –SH groups, where the percentage of dimers increased with increasing the pH from 5.8 to 9.5.[61]

Storage often leads to disulfide-based aggregation. Storage of a lyophilized excipient-free antibody (IgG1, containing 12 intra and 4 interchain disulfides) formulation for 1 year at 30°C resulted in formation of reducible dimer and trimers.[62] Similarly, reducible aggregates of a monoclonal antibody (type not specified) were detectable

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by SDS–PAGE after storage of the lyophilized formulation at 50°C for 6 months.[1] Such disulfide-linked covalent dimers and trimers were also observed during storage of spray-dried anti-IgE humanized monoclonal antibody.[71]

Nonreducible Cross-Linking

Nonreducible cross-linking was observed in several studies. Incubation of a human monoclonal antibody (C23) in solution at a basic pH at 37°C for 14 days resulted in formation of nonreducible bands above the light chains at pH 4 and 10.[72] Incubation of two mouse monoclonal antibodies—IgG2a (κ) and IgG1 (κ), between pH 3.0 and 10 at 37°C for 32 days led to formation of a nonreducible 88 kDa species by SDS–PAGE.[17] A species with the same molecular weight was observed after incubation of another monoclonal antibody, OKT3 (IgG2a), at 37°C for 2 months.[10,73] Further examination suggests that this species is a cross-linked product of H and L chains between the L46-52 and H99-121 regions. Although the exact chemical nature was not determined, an oxidative step is apparently involved as inhibition of oxidation delayed the formation of this species.[10,73]

A recent study shows that a nonreducible thioether bond can be formed between C223 of the heavy chain and the C-terminal Cys residue of the light chain in humanized IgG1 antibodies. This cross-linked product has a molecular weight of 75 kDa by MS but shows an apparent molecular weight of 92 kDa by SDS–PAGE.[74]

Nonreducible cross-linked products were also observed in solid dosage forms. Incubation of a lyophilized mouse monoclonal antibody [IgG2a (κ)] without lyoprotectant or in the presence of 5% sucrose, dextran, or hydroxypropyl-beta-cyclodextrin (HP-β-CD) at 56°C for 18 days led to formation of 100-kDa band under reduced conditions by SDS–PAGE.[59]

Deamidation

Antibody deamidation has been extensively reported in the literature. This common protein degradation pathway is so prevalent that purified antibody preparations may contain many deamidated forms. For example, several deamidated forms of recombinant humanized monoclonal antibody HER2 (rhuMAb HER2, Herceptin, trastuzuMAb) were found by cation-exchange chromatography, including (1) deamidated Asn30 (to aspartate) on one light chain, (2) deamidated Asn30 (to aspartate) on two light chains, and (3) deamidated Asn55 on the heavy chain (to isoaspartate).[37] The sole conversion of Asn30 to aspartate (not isoaspartate) suggests that the deamidation mechanism in cell culture may be different from that during storage.

Storage can easily generate large amounts of deamidated products. Incubation of a monoclonal antibody, OKT3 (IgG2a) in a PBS solution at pH 7 containing Tween 80 at 37°C for 2 months led to more than 90% deamidation at Asn H386.[10] By monitoring the ammonia generation, Poborji et al.[41] estimated that 4% of the total number of Asn and Gln residues in a chimeric antibody (L6) was deamidated after storing the solution at pH 7.2 at 50°C for 3 weeks.

Deamidation also occurs in solid state. In the evaluation of storage stability of a model recombinant humanized monoclonal antibody (rhuMAb HER2), Cleland et al. reported that incubation of a high-moisture formulation (8.4% moisture) for 12 months at 40°C resulted in a 19% drop in the main peak by IEX due to deamidation.[57]

Deamidation in proteins goes mostly through the intermediate of succinimide at Asn (more readily) and Gln (see reviews[75,76]).This seems to be the case for antibodies, even though alternative mechanisms were reported, such as deamidation of Asn30 on the light chain of recombinant humanized monoclonal antibody HER2 (rhuMAb HER2, Herceptin, trastuzuMAb).[37] A method has been developed to detect the formation of succinimide.[77]

The rate of deamidation via succinimidation in proteins can be affected by a variety of factors, such as pH, sequence, and steric effect (see review[44]). A recent report demonstrated a strong pH-dependency of deamidation at Asn55 (pH 7 > pH 6 > pH 5 > pH 4) in a fully human IgG1 in the pH range of 4–7 during storage at 25°C.[78] Using small peptide as a model, Tyler-Cross and Schirch[79] found that deamidation of Asn residues depends on the amino acid residue on the carboxyl side of Asn in neutral and alkaline solutions. Increasing the size and branching of these amino acids decreased the rate of deamidation by as much as 70-fold compared with the N–G sequence (greatest rate of deamidation) but under acidic conditions, these amino acids lost their protective effect.

Isomerization

The most common isomerization in antibodies is iso-aspartic acid formation, which results not only

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from direct isomerization of Asp but also, mostly, from hydrolysis of succinimide intermediate. The pH-dependent formation of succinimide intermediate can occur either from Asn deamidation or Asp dehydration.[36] In neutral and alkaline condition, the isoAsp and Asp products formed in a peptide were at a ratio of about 3:1.[79]

Isomerization is also prevalent and difficult to control. Again, many purified proteins contain significant amount of isomerized products, either from deamidation or direct isomerization. A typical example is recombinant humanized monoclonal antibody HER2 (rhuMAb HER2, Herceptin, trastuzuMAb).[37] In addition, since isomerization occurred at Asp H102 in CDR regions, the activity of the isomerized species is only 9–21% of the major species.[37] Similarly, isomerization of Asp L32 on one light chain in a recombinant monoclonal anti-IgE antibody (E25) reduced the relative binding activity of $F(ab)'_2$ to 42% and the activity of isomerized form on both chains (iso-Asp-iso-Asp) to 15%.[36]

Since formation of the succinimide intermediate does not need participation of water, it can readily occur in solid state. Asp isomerization is one of the major degradation pathways for a lyophilized, monoclonal antibody (type not specified), and increases with increasing storage temperatures both above and below their Tg values.[1]

Like deamidation, the relative rate of isomerization can be influenced strongly by steric effect. For example, a recombinant monoclonal anti-IgE antibody (E25) contains two Asp-Gly sequences within its CDRs, but only one site was found to be labile to isomerization (Asp L32). The unreactivity of one Asp residue is suggested to be due to hydrogen bonding.[36]

Oxidation

The oxidizable residues in proteins include Met, Tyr, Trp, His, and Cys. Although oxidation is not as prevalent as deamidation and isomerization in antibodies, it can easily occur during storage of antibodies. For example, the major route of degradation of a monoclonal antibody, OKT3 (IgG2a), in solution during storage at 5°C is oxidation of a nondisulfide Cys and several Met residues.[10] Oxidation was also observed in rhuMAb anti-CD20 in solution during storage at 40°C for 2 months.[55]

Oxidation of CDP870, a pegylated one-half antibody (one light and one heavy chain) at a Met residue (M3) was also observed after incubation of the protein at 25°C or 40°C for 12 weeks (14% at pH 4.1 and 4.2% at pH 5.5).[80] Light exposure can accelerate baseline oxidation. For example, light exposure (20,000 lux for 2 weeks at 27°C) of recombinant humanized monoclonal antibody HER2, rhuMAb HER2 caused 5–10% increase in oxidation in various liquid formulations at Met H255 (primary site) and Met H431 at temperatures 30 and 40°C.[81]

Formation of Acidic or Basic Species

Antibodies can form acidic or basic species, which are often monitored by IEF or ion exchange chromatography. Among all the possibilities, deamidation is the most likely degradation pathway, leading to formation of acidic species. Therefore, formation of such species is used loosely as an indication of deamidation in the absence of other confirmatory studies. Strictly speaking, formation of such species can only be considered as an indication of chemical instability (see below). A pH-dependency is almost certain for this kind of degradation. In the stabilization of CDP870, a one-half antibody (one light and one heavy chain), which is linked through Cys to a branched (20 kDa each) PEG, Johnson et al.[80] found that the protein generated more acidic species (up to 17% at pH 4.1 and 41% at pH 5.5) by IEF as the solution pH was increased from 4.1, 4.5, 5.0, to 5.5 during storage at 25°C for 12 weeks.[80]

Another possible mechanism for generating acidic species in antibodies is the Maillard reaction, where reducing sugars can react with lysine residues. Such a reation will result in loss of positive charges and formation of apparently more acidic species. This is the case for an spray-dried anti-IgE humanized monoclonal antibody with lactose during storage.[71]

Formation of basic species was also observed in antibodies. Its formation can result from several possibilities, most likely as a result of succinimide formation, removal of sialic acids, or pyroGlu formation. Such a basic species was observed after incubation of a human monoclonal antibody (C23) at 37°C for 14 days in a solution at an acidic pH by IEF.[72]

C-Terminal Clipping

Terminal cleavages can easily occur during normal production of antibodies. For example, the majority of a purified chimeric protein rCD4-IgG lacks the heavy chain C-terminal Lys.[82]

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This is also the case for recombinant humanized monoclonal antibody HER2 (rhuMAb HER2, Herceptin, trastuzuMAb)[37] and a recombinant monoclonal anti-IgE antibody (E25).[36] It was suggested that the cleavage was due to the action of basic carboxypeptidases during production.[37]

Fragmentation

The most likely sequences leading to fragmentation in proteins are Asp-Gly and Asp-Pro,[83] although other sequences can also experience cleavages, such as Asn-Ser.[79] Fragmentation can easily occur in antibodies even during production processes. A small amount of fragment (MW < LC) was detectable under reduced condition in a purified recombinant MAb IgG4 produced in Chinese hamster ovary (CHO) cells.[7] Half molecules should be monitored routinely for IgG4s.[84]

A variety of processing conditions may accelerate fragmentation of antibodies, such as acidic or basic treatment,[72] thermal treatment,[41,67] freeze-thaw,[41] and storage.[17,73,78] Fragments can simply include masses with a loss of a light chain (M-LC), a loss of a Fab arm (M-Fab), and separated heavy-chain (HC) and light-chain (LC), as well as species less than common antibody subunits, resulting from both peptide and/or disulfide bond cleavage.[67] Antibodies are also subject to radiation-induced cleavages.[85]

Maillard Reaction

Proteins may form adducts with sugars. In an investigative study, mouse MAbs were found to form glucose adducts after incubation with 0.5 M glucose at pH 7.4 for 14–21 days at 37°C and the glycated antibodies significantly increased the dissociation of the antibody-antigen binding complex.[86]

ANTIBODY FORMULATION

As mentioned above, antibodies, like other proteins, may generate a variety of degradants during production, processing, and storage both in liquid and solid states. Their tendency to generate such degradants depends very much on their individual sequence, pI, hydrophobicity, and carbohydrate content.[23] The antibody degradation products may have reduced activity and more importantly, increased immunogenicity.[14,87] A strong immune response (antibody formation) may lead to severe neutralization of the protein drug product and even endogenous proteins.

The major purpose of formulating antibodies as therapeutic agents is to control the rate of antibody degradation to provide an acceptable shelf life for worldwide shipping and storage. This can be done by choosing proper formulation excipients and other formulation conditions. It should be stressed that one formulation excipient stabilizing a specific antibody may not be suitable for another because of the differences in their sequence. A proper formulation composition should not only maximally preserve the activity of the antibody but also minimize the potential immune response, as excipients could alter the immune response by changing the presentation of the protein (such as the spacing of epitopes).[14] The following section will discuss critical factors in formulating both liquid and lyophilized antibody products. Advanced formulations will also be briefly described.

Liquid Formulation

Liquid dosage form is usually preferable to lyophilized products as it is easier to administer and less expensive to manufacture. Among all the commercial antibody products, about half are stable enough to be formulated in a liquid form (Tab. 1). Formulating a successful liquid product needs consideration of at least the following aspects.

Protein Concentration

Since the EC50 values of antibodies are often 10–1,000 times higher than for other proteins such as hormones and cytokines,[35] a relatively large amount of antibodies needs to be dosed to achieve any therapeutic effect. A large dose requires that an antibody product needs to be formulated/reconstituted at a high concentration to reduce dose volume, especially for subcutaneous injections; otherwise, a large volume of antibody preparation has to be infused. Since the aggregation process is usually concentration-dependent, antibodies at high concentrations have at least two potential issues: high tendency to aggregate during storage and likely high viscosity, leading to more difficulty during injection (injection time correlated with viscosity).[47] It has been suggested that the concentration-dependent protein aggregation is the greatest challenge to developing high-concentration

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protein formulations.[47] On the other hand, moderate increase in protein concentration may not have a significant effect. In fact, increasing the concentration of antifebrin T2G1s Fab′ (IgG1 (κ)), from 0.05 to 5.0 mg/mL in solution resulted in a decrease in dimer formation during storage (73.2% to 22.9% after 13 weeks at 22°C).[61]

An effective way to reduce the viscosity of an antibody solution is to add sufficient amount of NaCl.[5,88] Adjustment of formulation pH could potentially reduce the viscosity of an antibody solution.[88] Although not very effective, histidine has been shown to reduce the solution viscosity of a fully human anti-IL8 monoclonal antibody (ABX-IL8; IgG2) from 20 to about 9 centistokes when its concentration was increased from 0 to 60 mM.[21] However, use of such excipients in reducing viscosity can be limited, as the excipient can adversely affect the stability of the antibody, such as MAb anti-CD20.[55]

Another potential issue in formulating high-concentration antibody products is their solubility limits, as presence of undissolved proteins are generally not acceptable for intravenous injection. Fortunately, the solubility of antibodies seems to be relatively high and solubility-limited formulation difficulty has rarely been reported to our knowledge. To increase the solubility of antibodies, excipients such as surfactants may be used. In a recent report, Golovanov[89] found that a combination of L-Arg and L-Glu at an equimolar concentration (50 mM) significantly increased the solubility of several proteins (either alone does not have a significant effect). In addition, the room-temperature storage stability (fragmentation) of proteins was significantly improved as monitored by SDS–PAGE.

One potential advantage of formulating a protein product at higher concentrations is the greater freeze-thaw stability. Often, proteins at high concentrations are more resistant to freeze-thaw-induced aggregation, as the interface-induced protein denaturation can easily reach a saturation point. However, it does not seem to be the case for antibodies. Increasing the concentration of a chimeric antibody (L6) did not inhibit freeze-thaw-induced aggregation.[41] In addition, surfactants such as Pluronic F68 and Tween 80 did not appreciably prevent antibody aggregation. In a different study, Tween 80 alone (or Pluoronic F68) at 0.1% failed to offer any protection against freeze-thaw-induced aggregation of a single-chain antigen-binding protein CC49/218 sF_v (MW 27,000 d) at 1.45 mg/rnL in sodium PBS at pH 7.3.[56] These results suggest that freeze-thaw-induced antibody aggregation may not be a surface phenomenon.

Effect of Formulation pH

Formulation development often starts with determination of a stable formulation pH. Like other proteins, the pH effect on the stability of antibodies depends on the formulation composition,[55] stress conditions,[41] and even antibody concentration.[84] Formulation pH may affect the physical stability of antibodies as it alters the number and distribution of charges on the protein surface. For example, low pH (below 3) caused a loss of tertiary structure in rhuMAb anti-CD20.[55] Low pH (pH 3 and 4) is also responsible for the precipitation of two mouse monoclonal antibodies IgG2a (κ) and IgG1 (κ) during incubation at 37°C for 32 days.[17] Susceptibility to pH stress varies among different IgG molecules.[68]

Regarding chemical stability, formulation pH may play a critical role in controlling many degradation pathways, such as disulfide bond formation/exchange, deamidation, fragmentation, isomerization, etc. Due to variable effects on different degradation pathways, the optimum pH value varies depending on both the sequence of an antibody and the analytical methods used in monitoring the stability. By IEF, Lam et al. found that rhuMAb anti-CD20 is most stable at pH 5 within the pH range of 3–6 irrespective of protein concentration in formulations containing 10 mM citrate, 8% trehalose, and 0.05% Tween 20.[55] By SEC-HPLC, Usami et al. found that pH 6 was the most stable condition during incubation of a human monoclonal antibody (C23) at 37°C for 14 days in a solution containing isotonic sodium chloride between pH 4 and 10.[72] Sometimes, the optimum pH values reside outside a certain range. In the study of the stability of a chimeric antibody (L6), minimum amount of dimmer formation was observed at pH below 5.5 or above 8.5 (less than 2%) during freeze-thaw.[41]

It needs to be mentioned that oxidation could be inhibited by adjusting formulation pH. In a formulation containing 40 mg/mL MAb anti-CD20, 150 mM trehalose, 0.9% benzyl alcohol, 0.02% Tween 20, and 50 mM histidine, reducing the pH from 7.5 to 5 virtually eliminated the oxidation during storage at 40°C for 2 months.[55] In a different study, oxidation (M3) of PEG-CDP870, a one-half antibody, in a formulation containing 160 mg/mL CDP870, 10 mM sodium acetate, and

125 mM NaCl was slower at pH 5.5 than at pH 4.1 (4.2% vs. 14% after 12 weeks at 40°C).[80]

Due to the simultaneous presence of multiple antibody degradation pathways, the formulation pH has to be adjusted to balance all the potential degradations. Generally, a weakly acidic condition seems to be the optimum for most antibodies (see Tab. 1).

Effect of Buffering Agents

It has been long recognized that both the type and concentration of a buffering agent may affect the stability of proteins (see review[44]). For example, phosphate buffer accelerated deamidation of an IgG1 relative to citrate buffer at both pH 6.5 and 7.0 during storage at 25°C.[78] Therefore, proper choice of a buffering agent can potentially achieve both pH control and stabilization of antibodies. In rare cases, a buffering agent good for one antibody may be harmful to another. One of these agents is histidine, which has a pKa of pH 6 and potentially good in controlling a weakly acidic condition. It has been shown to provide better protection against precipitation for a fully human anti-IL8 monoclonal antibody (ABX-IL8; IgG2) upon thermal incubation both at 50 and 40°C than citrate at pH 6.[21] Addition of a small amount of histidine (0.4 mM) also increased the freeze-thaw (4×) recovery of a single-chain antigen-binding protein CC49/218 sF_v (MW 27,000 d) at 1.45 mg/rnL in PBS solution at pH 7.3 from 37% to 90%.[56] On the contrary, histidine was found not only to increase the rate of aggregation of rhuMAb anti-CD20 during storage but also to induce coloration of the solution compared to a acetate-buffered antibody solution at pH 5.[55] In addition, oxidation of Fc was also faster in histidine solution.

The concentration effect of a buffering agent was demonstrated in a study where freeze-thaw-induced aggregation of a chimeric antibody (L6) was higher with increasing phosphate buffer concentration (0.01 vs. 0.05).[41] Therefore, both the type and concentration of buffering agents need to be examined.

Effect of Formulation Excipients

Use of formulation excipients remains a major and convenient approach in stabilizing antibodies in solutions. It is so at least in reducing antibody aggregation.[47] A variety of formulation excipients have been shown to stabilize antibodies under different processing conditions and during storage, including sugars,[41] polyols,[41] amino acids,[41,84] surfactants,[84] and polymers.[45,84]

Formulation excipients are effective generally in protecting the physical stability of an antibody. Among these excipients, sugars are the most commonly used. A variety of sugars, such as sucrose, trehalose, glucose, have been shown to reduce the freeze/thaw-induced aggregation of rhuMAb anti-CD20,[55] a chimeric antibody (L6),[41] a human anti-IL8 monoclonal antibody (ABX-IL8; IgG2),[21] and a single-chain antigen-binding protein CC49/218 sF_v (27 kDa).[56] Other excipients can be equally or even more effective in protecting antibodies. Histidine was shown to be equally effective as sucrose in reducing freeze-thaw induced aggregation of a human anti-IL8 monoclonal antibody (ABX-IL8; IgG2) and combination with Arg resulted in a better protection than sucrose in solution stability at different incubation temperatures.[21] Sugars are not always effective. In the evaluation of freeze-thaw stability of a single-chain antigen-binding protein CC49/218 sF_v (MW 27 kDa) at 1.45 mg/mL in sodium phosphate buffered saline (PBS) at pH 7.3, addition of 10 mM trehalose, lactose, glucose, or mannitol did not offer any protection.[56] In a rare case, sucrose has been shown to promote agitation-induced aggregation of an IgG1 antibody.[90]

Formulation excipients may inhibit chemical degradation of antibodies. Replacing 0.14 M NaCl with 8% trehalose or 4% mannitol in a rhuMAb anti-CD20 increased the chemical stability as measured by IEX during storage at 40°C.[55] Such an effect is likely a result of stabilization of antibody conformation. Some excipients may protect antibodies against oxidation. Replacing NaCl with 4% mannitol and 1% benzyl alcohol protected recombinant humanized monoclonal antibody HER2, rhuMAb HER2, against oxidation (primarily Met-255) at 30 or 40°C.[81] This effect is likely due to a function of mannitol as a free radical scavenger. Some commonly used antioxidants, such as thiosulfate and methionine, are effective in inhibiting antibody oxidation. The minimum effective levels (molar ratios of antibody to antioxidant) required to inhibit temperature-induced oxidation of rhuMAb HER2 were 1:5 and 1:25 for methionine and thiosulfate, respectively.[81] However, use of thiosulfate has led to formation of a thiosulfate adduct of rhuMAb HER2.[81] The safety of such a thio-containing adduct has not been reported.

One of the main excipients often used in antibody formulation is surfactants. They are usually

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effective in reducing shaking/stirring-induced aggregation[91] but may have negative effect during long-term storage.[92] One obvious concern is that such compounds may contain a residual level of peroxides, which have been shown to cause protein oxidation.[93] Therefore, use of surfactants should be kept to a minimum in an antibody formulation if possible. For example, removing Tween 20 in a recombinant humanized monoclonal antibody HER2, rhuMAb HER2 solution reduced the oxidized antibody from 52% to <10% at 40°C.[81]

Effect of Shaking/Shearing

Both shaking and shearing can denature antibodies. It was found that shaking murine antibody-based products caused antibody precipitation and the shape of the precipitates are different depending on the fill volume.[53] In the evaluation of shear effect on the antigen-binding activity of a recombinant scFv antibody fragment in a buffer solution, Harrison et al.[45] found that the binding activity decayed at a mean rate constant of 0.83/h at a shear of approximately 20,000/s.

Effect of Preservatives

A limited number of studies have been conducted on the compatibility of preservatives with antibodies. Compatibility depends both on the antibody and the preservative. In the evaluation of the effect of several preservatives, including benzyl alcohol, chlorobutanol, methylparaben, propylparaben, phenol, and m-cresol, on the stability of a humanized monoclonal antibody, Gupta and Kaisheva[94] found that the antibody was most stable in the presence of methylparaben and propylparaben, compatible with benzyl alcohol and chlorobutanol at low concentrations, and not compatible with phenol and m-cresol, as measured by a variety of assays, including SEC-HPLC, DSC, light scattering, UV, and potency assay.

Effect of Processing Equipment

Another important issue in antibody formulation is the choice of proper processing equipment/containers. A few studies have shown that antibody stability can be significantly affected if an inappropriate choice is made. In the study of the stability of recombinant humanized monoclonal antibody HER2, rhuMAb HER2, in liquid formulations, Lam et al.[81] found that replacing the stainless steel filler with a nonstainless steel filler reduced the oxidized antibody (primarily Met-255) from 52% to 18% after storage for 2 weeks at 40°C. It was suggested that Fe ions, released from erosion of stainless steel by chloride ion at low pH, catalyzed the oxidation. In a similar case, Chen et al.[21] demonstrated that a fully human anti-IL8 monoclonal antibody (ABX-IL8) in aqueous formulations containing 60 mM His had a higher level of aggregates after freeze-thaw in a stainless steel vessel and subsequent storage at 40°C, possibly due to the effect of Fe ions generated from a similar mechanism. In addition, proteins have been shown to facilitate metal corrosion.[95]

Effect of Product Containers

The effect of product containers was clearly demonstrated in a recent study. McCormick et al.[96] reported that Tween 80, intended to replace human albumin in the original formulation, interacted with uncoated rubber stoppers in single-use Eprex syringes. The stoppers leached a small amount of plasticizers in the drug solution, which acted as an adjuvant, and caused a strong IgG response to the recombinant human erythropoietin. The immune response was significantly minimized later by switching to PTFE-coated rubber stoppers. In addition, headspace oxygen can accelerate oxidation of antibodies and replacement with an inert gas could minimize such a reaction.[73]

Accelerated Stability Studies

To accelerate formulation development process, accelerated stability studies are usually conducted. However, estimation of room temperature or 5°C stability based on accelerated stability studies for antibodies, like other proteins, may or may not be possible or accurate. The unpredictability is due to the presence of complex and multiple degradation pathways, which may have different degree of temperature dependencies.[10] Kroon et al.[10] found that relative to other degradation pathways of a monoclonal antibody, OKT3 (IgG2a), Asn deamidation was preferentially accelerated with increasing temperature. Therefore, caution needs to be applied in interpreting such high-temperature-induced deamidation. On the other hand, Poborji et al.[41] found that loss of monomeric chimeric antibody (L6) at pH 7.2 followed Arrhenius behavior in the temperature range of 30–50°C and extrapolation of the high temperature SEC data to 2–8°C at pH 7 and

5.5 resulted in comparable stability estimates with the available real-time data.

Lyophilized Formulation

Preparation of lyophilized protein formulations has been extensively reviewed.[97–101] Like most proteins, some antibodies are not stable enough in a liquid form and lyophilized dosage forms will have to be considered. Lyophilization was suggested to be one of the two approaches in solving antibody aggregation.[47] This section will discuss some critical issues in formulating a lyophilized antibody product.

Amorphous Versus Crystalline State

It has been commonly believed that proteins are more stable in an amorphous state.[103] This is mainly because proteins need another excipient(s) to replace water molecules to from the required H-bonds as water molecules are removed during drying and a crystalline matrix does not provide intimate H-bonding between the excipient and the protein.[104,105] It appears that if the protein itself can be crystallized during the lyophilization process (may be difficult practically), the protein could be more stable and might not need as much stabilizing agent as in the amorphous state. In a recent report, Shenoy et al.[106] compared the stability of two model proteins, glucose oxidase (GO) and lipase, and demonstrated that dry crystalline formulations are significantly more stable than their amorphous counterparts as monitored by size-exclusion chromatography. It remains to be seen whether this can be applicable to antibodies.

Effect of Formulation Excipients

To make a lyophilized antibody product, at least one formulation excipient, a bulking agent, is needed to confer an acceptable cake. If the antibody is not stable enough in the presence of a bulking agent during lyophilization or storage, a stabilizer(s) is included. The relative amount of formulation excipients (bulking and stabilizing agents) should be carefully chosen as these agents may affect each other's behavior, the tonicity, and stability of the lyophilized products.

Commonly used bulking agents include mannitol and glycine. Crystallization of these agents during lyophilization makes them wonderful bulking agents but poor stabilizing agents.[57] Although mannitol could stabilize an antibody as in the case for a monoclonal antibody-vinca conjugate in a PBS solution at pH 7.4 upon lyophilization,[60] many studies showed that mannitol could not stabilize effectively or even destabilizes antibodies. Such examples include the aggregation of a lyophilized model recombinant humanized monoclonal antibody (rhuMAb HER2) in the presence of mannitol during storage.[3,57] In the lyophilization of a human monoclonal antibody at 1 mg/mL, it was demonstrated that addition of 5% mannitol has no effect on the lyophilization-induced aggregation in PBS or physiological saline as measured by OD600 and addition of 10% mannitol actually accelerated aggregation during the lyophilization process.[58] On the other hand, when used in combination with sucrose or trehalose, similar degree of stabilization to that for sugar alone may be achieved such as the case for model recombinant humanized monoclonal antibody (rhuMAb HER2).[57] The disaccharide/mannitol formulations also inhibited deamidation of this antibody during storage to a greater extent than the lyoprotectant formulations alone.[57]

Sugars are the most common stabilizing agents used in lyophilized formulations. Among these, two nonreducing sugars, sucrose and trehalose, are usually the first choice. Both sugars protected the stability of a few lyophilized antibodies equally well. These antibodies include lyophilized recombinant humanized monoclonal antibody, rhuMAb HER2,[57] IgG1 (containing 12 intra and 4 interchain disulfides),[62] anti-IgG,[107] and chimeric monoclonal antibody.[64] However, sucrose seemed to be more effective in protecting a lyophilized IgG1 against aggregation during storage at 40°C at a sugar:protein weight ratio of 1:1 (Chang et al. 2005b).[102] In another study, sucrose at 5% failed to offer any protection to a lyophilized mouse monoclonal antibody (MN12) (IgG2a (κ)) during storage at 56°C for 18 days.[59] Again, as mentioned in the liquid formulation section, even the most commonly used stabilizers may not stabilize all antibodies in the solid state and antibodies need to be treated as individually different molecules.

When sugars are used as stabilizing agents (lyoprotectants), a sufficient quantity needs to be used to achieve a significant effect depending on the protein concentration. It has been suggested that a molar lyoprotectant:protein ratio of 300:1 or greater should be used.[47] Indeed, the available literature data indicate a ratio higher than 300 is needed for better stabilization. It was shown that sugars protect the stability (aggregation and Asp isomerization) of a lyophilized, monoclonal

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antibody (type not specified) to a significant level when the sugar to protein molar ratio was increased to 500:1, which happens to the theoretical number of water binding sites in the molecule.[1] Similarly, Andya et al.[62] demonstrated that use of sucrose or trehalose inhibited the aggregation of a lyophilized antibody (IgG1—containing 12 intra and 4 interchain disulfides) during storage at 30°C to a maximum degree when the sucrose/trehalose:protein ratio was 500 or higher, which is roughly equivalent to the number of water-binding sites on the surface of the protein (550 estimated). Increasing the trehalose/anti-HER2 ratio from 60 mM (360 molar ratio) to 200 mM (1,200 molar ratio) significantly increased the stability (monomer by SEC-HPLC) of lyophilized formulation at 25 mg/mL at 40°C.[107] In a recent report, it was found that at least a weight ratio of 2:1 (molar ratio of about 800) was needed to achieve a maximum protection of a lyophilized sucrose:IgG1 formulation against both aggregation and chemical degradation during storage at 40°C (Chang et al. 2005a).[101]

Other formulation excipients were also used and some of these are effective in stabilizing certain antibodies. A recent study demonstrated that addition of sorbitol to a sucrose or trehalose formulation resulted in further enhancement of storage stability of a lyophilized IgG1 antibody (Chang et al. 2005b).[102] Either dextran or HB-β-CD (better) protected the activity of a lyophilized mouse monoclonal antibody (MN12) (IgG2a (κ)) during storage.[59] Glucose was shown to inhibit gamma irradiation-induced fragmentation of humanized monoclonal antibodies (hr3).[85]

Effect of Formulation "pH" and Buffering Agents

The pH of the liquid formulation can have a potential effect on the stability of the lyophilized formulation, even though the solid-state "pH" could not be well defined. In the evaluation of the stability of a lyophilized monoclonal antibody-vinca conjugate, aggregation seems to be lower at a neutral pH 7.1 than at either pH 8.5 or 6.1 when the lyophilized formulation was stored at 25°C.[60] In adjusting the formulation pH, use of a buffering agent may have effect on the antibody stability both during lyophilization and storage of the lyophilized products. For example, in the stability study of a fully human anti-IL8 monoclonal antibody (ABX-IL8; IgG2), Chen et al.[21] demonstrated that addition of histidine inhibited the formation of HMW species upon lyophilization and during storage at 37°C in a concentration-dependent manner. Among several excipients including His, Gly, mannitol, Glu, and polysorbate 20, His is the most critical in protecting solid-state stability of the antibody (minimizing aggregation). In a different study, the aggregation rate of a lyophilized anti-IgE at 5°C at 5 mg/mL in 10 mM buffer is affected both by pH and buffers in the following order: Na Phosphate, pH 7 > K Phosphate, pH 7 > Na Succinate, pH 6 or 5 > Histidine, pH 7.[107] It should be noted that some buffering agents may induce significant pH change due to selective crystallization during lyophilization, such as sodium phosphate. Such buffering agents should be avoided in preparing pH-sensitive antibody formulations.

Effect of Protein Concentration

In many cases, increasing the protein concentration increases the stability of the protein during lyophilization (see review).[98] However, antibodies do not seem to follow this trend and many antibodies have been shown to be less stable both during lyophilization and storage at high concentrations. For example, lyophilization of a human monoclonal antibody caused significant aggregation of MAb formulated in PBS or physiological saline as measured by OD600 and increasing the protein concentration from 1 to 2.5 or 5 mg/mL increased the OD600 from 0.194 to 0.311 or 0.427, respectively.[58] Similar trend was also observed during storage of lyophilized formulation. Increasing the concentration of a chimeric monoclonal antibody from 2.5 mg/mL to 25 mg/mL in a lyophilized formulation, containing 31 mg/mL sucrose, 15 mM NaCl, 0.02% Tween 80, 10 mM citrate, increased the aggregate level from 0.4% to 0.6% after 2 months at 5°C.[64] In the evaluation of the stability of a model recombinant humanized monoclonal antibody (rhuMAb HER2), Cleland et al.[57] demonstrated that increasing the protein concentration from 25 mg/mL to 50 mg/mL in a lyophilized formulation (containing 60 mM trehalose, 5 mM His at pH 6) increased the aggregate level from 8.5% to over 30% by SEC-after storage at 40°C for 44 months. Such behavior was also observed of anti-HER2 from 5 to 21 mg/mL containing 250 mannitol.[107]

Effect of Moisture Content

The effect of moisture on the stability of lyophilized antibodies has not been consistent and seem

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to depend on the antibody and the degradation pathways being monitored.[57] Generally, increasing moisture content of a lyophilized antibody will gradually increase the rate of degradation during storage. Such negative effect of moisture was clearly demonstrated in a variety of degradation pathways, including dimerization of T2G1s Fab′ (IgG1 (κ)) in a moisture content range of 1.5–12.3%,[61] aggregation of TNF-MAb between 0.68% and 8%,[108] formation of acidic/basic species of rhuMAb HER2 between 1% and 8.4%[57] and fragmentation of a mouse monoclonal antibody (MN12) with and without secondary drying.[59]

In other cases, the effect of moisture is either complex or not significant. For example, Breen et al.[1] found that high-moisture formulations (8%) of a lyophilized monoclonal antibody (type not specified) showed a higher rate of aggregation, fragmentation, and Asp isomerization during storage than those containing 2% or 5% moisture but formulations containing intermediate moisture levels (2–3%) were less prone to aggregation than those containing a lower moisture levels (1%) as measured by SEC under accelerated storage conditions. Similarly, the aggregation of a lyophilized IgG1 was found to be minimal at an intermediate moisture level around 3% at different storage temperatures. (Chang et al. 2005b).[102] In a different study, Cleland et al.[57] found that using a model recombinant humanized monoclonal antibody (rhuMAb HER2), a residual moisture between 1 and 8.4% had no impact on the rate of aggregation during storage at 2–8 or 40°C for 12 months.

Accelerated Stability Studies

Like antibodies in a liquid state, those in a solid state may or may not undergo degradation that is characteristic of Arrhenius behavior. It is generally believed that stability studies should be conducted at least below the glass transition temperature of the formulation. Duddu et al.[64] found that accelerated stability data generated in the glassy state (40°C) seems to be a better predictor of the relative stability of formulations than the data generated at a higher temperature (60°C). In the study of the stability of a high-concentration, lyophilized, monoclonal antibody (type not specified), Breen et al.[1] demonstrated that chemical and physical degradation pathways followed Arrhenius kinetics during storage in the glassy state. Only Asp isomerization followed the Arrhenius model above the Tg value.

Advanced Formulations

In addition to the liquid and lyophilized dosage forms on the market, other dosage forms have been tried and limited success has been reported.

Spray-Dried Formulations

Spray drying is an efficient way of preparing solid dosage forms because of the rapid removal of a solvent(s) from a drug solution. It is a very attractive alternative to lyophilization in preparing solid protein formulations. So far, a number of studies have been conducted for proteins and partly because of the high-temperature exposure in the process, albeit very short, limited success has been achieved. Protein aggregation during spray drying seems to be the major challenge.[65,66,109,110]

Spray drying of antibodies has also been tried. As for other proteins, process-induced protein aggregation seems to be the major issue.[71,110] Careful selection of proper excipients could minimize this problem. In the evaluation of a spray-dried formulation for a rhuMAbE25, it was demonstrated that trehalose or lactose minimized the aggregation during spray drying (aggregate dropped from 5–6% to ≤1%). For trehalose, the molar ratio for significant inhibition of aggregation is at or above 300:1 to 500:1 (excipient: protein).[71] Such a ratio seems to be comparable with that used in the lyophilized formation. The addition of mannitol also reduced aggregation, but only up to a molar ratio of 200:1 and further increase in mannitol content resulted in crystallization, which had a detrimental effect on protein stability and aerosol performance.[71] In a different study, either trehalose or sorbitol could inhibit IgG aggregation during spray drying.[110]

Stabilized Antibodies via Mutagenesis

Antibody fragments, such as scFv, are alternative antibody therapeutics. However, since their stability is not as good as full-length antibodies, mutagenesis, or chemical modifications have been tried to improve its stability. For example, R71A mutagenesis in the heavy chain of an unstable scFv dramatically increased its stability when incubated in human serum while having only a minor effect on the binding affinity of the molecule.[111] A more stable heavy chain, made through mutation, can increase the stability of

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the light chain, and thereby confer more stability to the whole scFv.[24]

Stabilized Antibodies via Chemical Modifications

The clinical performance of an antibody can be desirably changed by conjugation of the antibody to another entity. For example, McIntosh et al.[112] demonstrated that conjugation of carboxymethyl dextran to a tumor-antigen-specific monoclonal antibody (A5B7) increased the clearance of the antibody in mice but conjugation of cisplatin (*cis*-dichlorodiammine platinum II) with the antibody complex restricted its tissue distribution and reduced its systemic clearance.

PEGylated Antibodies

A common method of increasing plasma half-life of proteins is to increase the size of the protein by attaching a PEG moiety. Currently, there are at least four PEGylated commercial protein products on the market. However, the long plasma half-lives (as high as 7–23 days) of antibodies made much less appealing the development of such an antibody product. Since the long half-life was due to the binding of Fc to the Fc-receptor (neonatal Fc receptor, FcRn) in various tissues,[33,35] antibody fragments, such as Fab′ lacking the Fc portion have a much shorter half-life and could be good candidates for PEGylation. Such a modification was made on a humanized IgG Fab′ fragment via site-specific attachment to an additionally engineered Cys residue, as modification via the lysine residues led to substantial losses in binding activity.[29]

Controlled Release Systems

Controlled release systems have been extensively investigated for proteins in the past two decades.[113,114] However, limited work has been conducted in this area for antibodies, partly due to their relatively long plasma half-lives compared with other proteins. To achieve long-term effect, a controlled released system consisting of biodegradable poly (lactic-glycolic) acid (PLGA) was prepared for an anti-cocaine catalytic monoclonal antibody and an *in vivo* release of the antibody for up to 10 days was demonstrated in mice following subcutaneous injection.[115] It is anticipated that such systems could be useful if a minimum of 1-week duration of release is achieved.

SUMMARY

Antibodies have similar tertiary structures. The presence of a significant number of disulfide bonds and intimate domain–domain interactions in antibodies make them relatively stable and more resistant to moderate thermal stress compared to other proteins. Nevertheless, antibodies do experience a variety of instabilities similar to most proteins. These physical and chemical instabilities include denaturation, aggregation, surface adsorption, deamidation, oxidation, isomerization, fragmentation, etc. Due to the significant difference in the primary sequence among different antibodies, the relative severity of these degradation pathways can be significantly different. On the average, aggregation, deamidation, and isomerization seem to be more prevalent in antibodies.

Partly due to the stability difference, some antibody products are made into a liquid form while others are lyophilized (Tab. 1). The solution pH of these commercial products is either neutral or weakly acidic, suggesting that such a pH range is probably optimal for most antibodies. Surfactants have been used in most of these antibody products, presumably to inhibit antibody aggregation, and will likely be used in other antibody drug candidates. Two most commonly used formulation excipients in these antibody formulations are sucrose and NaCl. For liquid formulations, either one can be used if they offer the same level of stability protection. For lyophilized formulations, sucrose is preferable as NaCl does not form intimate H-bonds with the antibody and also reduce the glass transition temperature of formulation, making lyophilization less efficient. All the formulation excipients and buffering agents used in commercial antibody products or discussed in the article should be evaluated individually for each antibody drug candidate through stability studies before they are chosen as part of the product, as antibodies are structurally different.

The available stability data in the literature and the information on the commercial antibody products suggest that antibody formulation seems challenging but relatively manageable, compared to other protein drugs. Three major issues in antibody formulation are apparently challenging and need significant attention in the coming years: (1) development of stable high-concentration formulations, (2) management of the high-concentration-associated viscosity of these formulations,

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and (3) minimization of antibody-formulation-induced immune response.

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Formulation and delivery issues for monoclonal antibody therapeutics☆

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Abstract

Antibodies can have exquisite specificity of target recognition and thus generate highly selective outcomes following their systemic administration. While antibodies can have high specificity, the doses required to treat patients, particularly for a chronic condition, are typically large. Fortunately, advances in production and purification capacities have allowed for the exceptionally large amounts of highly purified monoclonal antibodies to be produced. Additionally, genetic engineering of antibodies has provided a stable of antibody-like proteins that can be easier to prepare. Together, these advances have made antibody-based therapies one of the most commonly pursued pharmaceuticals in biotechnology pipelines. With this success, however, has come a series of technical challenges in the formulation of antibody-based materials to maintain sufficient stability in a variety of configurations and sometimes at particularly high concentrations. This review focuses on issues related to identifying and verifying stable antibody-based formulations.
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Keywords: Antibody; Stability; Delivery; Formulation

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1. Introduction

Therapeutic and diagnostic antibodies have become the fastest growing area of biopharmaceutical applications. There are now 18 monoclonal antibodies on the market and over 100 in clinical trials, which highlights the significance of these therapeutics and the advances made in antibody engineering. Further, by 2008, engineered antibodies are projected to be the source of over a third of the revenues from biotechnology [1,2].

A few seminal events have led to the current and projected prominence of antibodies as biopharmaceuticals include the identification of methods to generate murine monoclonal versions of antibodies [3], the cloning of human antibody sequences to allow for humanization of murine monoclonal antibodies through complementary-determining region (CDR) grafting [4] and even the establishment of fully humanized systems to generate monoclonal antibodies [5]. With the sequential identification of these technological advances, antibodies for therapeutic and prophylactic indications have moved from fully murine, to partially murine (mostly human) to completely human constructions. Added to these events, dramatic advances in production have led to the ability to prepare monoclonal antibodies at scales that can provide sufficient material at costs that make this area appealing to pharmaceutical companies. One important outcome of these various advances is a greater potential to use antibody drugs in chronic settings, tremendously expanding their biopharmaceutical applications.

The majority of currently approved antibody drugs address previously unmet medical needs in the areas of cancer and inflammation. These indications came from practical considerations related to earlier forms of murine and partially murine antibody therapeutics; the first monoclonal to be approved (OKT-3®, muromonab-CD3) was approved in 1986 for a single treatment regimen to protect from life-threatening tissue rejection following kidney transplant [6]. Adverse reactions due to acute immunogenicity problems due to delivery of a non-self protein limited the use of OKT-3® to only acute application [7,8]. Subsequent partially murine antibodies such as Panorex® (17-1A), Zevalin® (ibritumomab tiuxetan) and Bexxar® (tositumomab) were approved for a limited number of administrations to cancer patients who had failed frontline therapies [2]. With the advent of fully humanized antibodies, chronic therapeutic or prophylactic applications for infectious agents have become a realistic clinical option [2]. Another critical aspect to the success of antibodies as therapeutic agents involves improved methods to express, purify and characterize these proteins [9]. In general, antibody therapeutics are

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large (typically >150 kDa), complex in nature (most are glycosylated) and must be administered in stoichiometric rather than catalytic quantities (nearly a gram per dose is required for many antibodies to be effective). Production and purification scales have thus reached levels of production that were previously assumed impossible in biotech companies [10]. Novel concerns then related to the development of stable formulations and delivery strategies for such large amounts of a complex molecule. Understanding product instability with regard to physicochemical and thermodynamic events becomes more and more critical to ensure market success [11]. The required high concentrations of antibodies in these formulations reduced the need to add a carrier protein, as is the case where serum albumin is commonly added in formulations of highly potent proteins such as erythropoietin alpha (Epogen®) and Factor VIII (Kogenate®) to enhance stability and reduce loss to vial surfaces. Simultaneously, the large amounts of an antibody protein used in these formulations allowed degradation products to be more readily detectable particularly when using the highly sensitive methods designed for evaluating hormone biotherapeutics.

Finally, the ability to prepare large quantities of an antibody that could be administered for chronic therapy resulted in a plethora of new and novel applications [9,10] involving the delivery of chemotherapeutics, radionuclides and imaging agents — to name a few. New issues, related to identifying stable formulations such as high concentration formulations that might reduce the volume of antibody administrations, arose in the move from intravenous infusions to the realm of subcutaneous injection strategies. In some cases the combination of agents with antibodies produced conflicting stability profiles. Additionally, potential new indications for antibody drugs increased the desire to find novel methods for sustained release, intracellular targeting [12] or delivery via methods not involving an injection. Thus, as is the case in most scientific advances, technological advances opened up possibilities for therapeutic opportunities that were not possible previously as well as a totally new set of problems that required solving. In this review we have attempted to identify the major issues associated with formulating and delivering antibody drugs, to recognize challenges already identified accompanied when possible by solutions, as well as to discuss concerns that will need to be addressed in the future as the use of antibody drugs continues to evolve.

2. Antibody characteristics relevant for formulation and delivery

The human body produces several classes of antibodies that appear to perform specific functions under different conditions and at discrete anatomic sites. Most of the currently approved antibody-based drugs are of the IgG1 class [13] and are glycosylated on the Fc (fragment crystallizable) domain. Glycosylation has been shown to participate in specific cell response events mediated by antibodies [14–16]: antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cell-mediated cytotoxicity (CDCC) through receptor recognition events. Individual glycoforms of antibodies can provide improved efficacies for certain clinical outcomes [17]. In general, IgG1 and IgG3 antibodies are effective at initiating ADCC while IgG2 readily fixes complement but is poor at inducing ADCC. IgG4 appears to be inefficient at both ADCC and CDCC [13]. Interestingly, antibodies can be re-engineered to increase their capacity to recruit complement [18] or to adjust their affinities to the various classes of Fc receptors and thus ADCC function through mutation of specific amino acids within the Fc domain [19]. Molecular engineering has also provided a spectrum of new antibodies (Fig. 1). Other chapters in this issue delve deeply into the many characteristics of antibodies that make them remarkable and diversely applicable treatment agents: ligand binding properties, effector functions and potential for modification in their design. Other chapters will also address applications of newly engineered antibody-like molecules that move beyond the realm of intact, full-length IgG antibodies. In the current review, attention will be focused primarily on IgG1 since this is where the majority of published information exists for antibody drug formulation and delivery.

Antibodies, due to their large size (being greater than the filtration cut-off size of the kidney) and their ability to be sequestered for periods of time from the blood, via interactions with FcRn receptors present on

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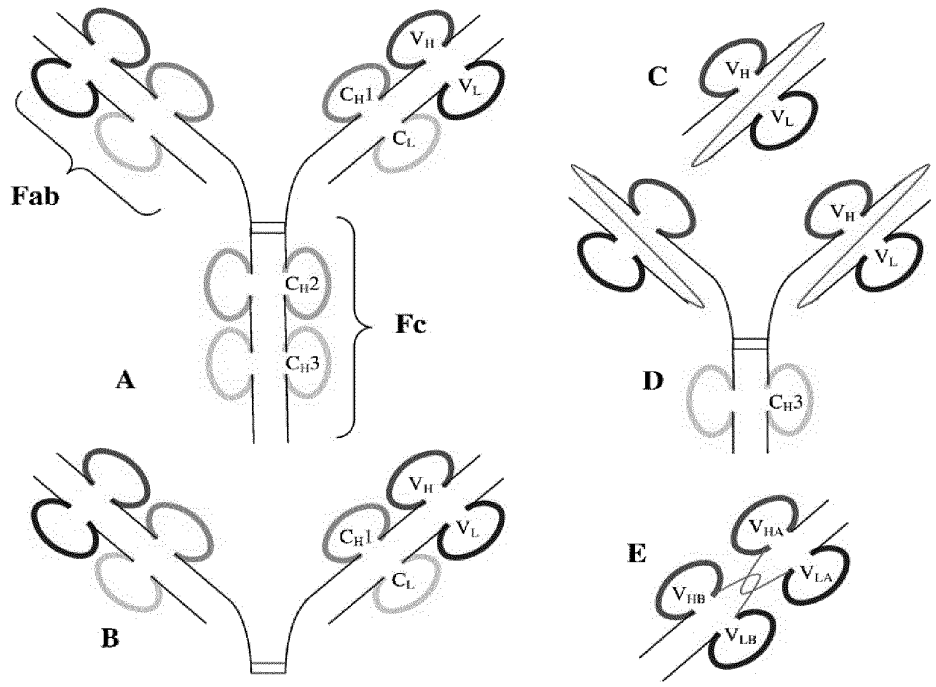


Fig. 1. General antibody structure and some antibody-based protein constructions. A) Human antibodies have a common framework that can be described as Fc (fragment crystallizable) and Fab (fragment antigen binding) domains that are derived from the folded structure of heavy (H) and light (L) chain proteins. Multiple constant (C) regions on both the H and L chain proteins are donated as are the variable (V) segments involved in antigen-specific recognition. B) Removal of the Fc domain produces a F(ab)2 structure. C) Single chain structures of the antigen binding region (scFv) can be prepared by themselves or D) in a construct that includes Fc domain elements. E) Bi-specific antibody elements (diabodies) can be prepared to bring two different antigens into close proximity. C_H, heavy chain constant regions; C_L, light chain constant region; V_H, heavy chain variable region; V_L, light chain variable region.

endothelial cells, can have extended serum profiles in the range of weeks. A number of engineered antibody-like molecules are now being examined for therapeutic applications [20]. Some engineered fragments, such as diabodies and minibodies, are much smaller than intact, full-length IgG1 and have very different properties of systemic distribution and clearance. Additionally, methods to prepare other antibody types such as dimeric secretory IgA [21] and methods to prepare antibodies using non-mammalian cell systems, such as plants [22], from the eggs of chimeric chickens [23] and from transgenic animals [24], are now being explored by academic and industrial groups. As these new approaches gain prominence, more information will be acquired about their unique requirements for successful formulation and delivery. At present, however, there is little information on formulation approaches for antibodies produced using these production processes.

3. General concerns for antibody formulations

Due to their proteinacous nature, antibodies present generic formulation issues that are similar for most protein therapeutics [11]. Proteins, in general, can be degraded under conditions when they are exposed to heat, freezing, light, pH extremes, agitation, sheer-stress, some metals and organic solvents. With ever advancing analytical technologies, the ability to assess the physicochemical and thermodynamic instability of antibody drugs has also led to the identification of several events that are more specific to the unique nature of this particular class of proteins: variations in

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Fc glycosylation, partial heavy chain C-terminal Lys processing, Fc methionine oxidation, hinge-region cleavage and glycation of Lys residues [25]. In addition to these issues is the difficulty of preparing dosing materials that might have protein concentrations in the range of 100 mg/ml or greater. Since early preclinical assessment is so critical to successful identification of a new biotherapeutic product, general pre-formulation strategies are usually employed for initial studies with new antibody drugs. An initial formulation effort will likely employ an isotonic, non-hemolytic, slightly acidic solution that can be administered by intravenous infusion or subcutaneous injection in studies employing a relevant animal model. In some cases, some stability information for the antibody drug can be obtained from initial in silico and in vivo testing during this pre-clinical phase that aids in the selection of a formulation that is used in clinical trials [26].

Since most early pre-clinical studies attempt to emulate the delivery strategy that will be used in the clinic, initial studies frequently identify problems with either the initial pre-formulation approach and/or stability issues with the antibody drug itself. In the case of the formulation, protein precipitation can occur with refrigerated storage or with repeated freeze–thaw cycles; events that may be facilitated by an inappropriate pH or buffer selection. Analysis of antibody drugs stored in this initial pre-formulation system may also identify unstable sites within the protein or its linkage to attached molecules (chemotherapeutics, imagining agents, etc.). In the case of the antibody, this information is frequently evaluated to determine if re-engineering of the protein could be performed, for example, to provide greater thermodynamic stability or folding efficiency [27]. Such changes in the Fc region are typically conservative, but might act to modify Fc receptor binding properties to provide a different biological outcome [28,29]. Amino acid substitutions can also be made within the Fv region to improve thermal stability [30], but such changes might also alter antigen binding specificity [31].

4. Stability issues for antibody formulations

If one examines the amino acid sequences for currently approved IgG1-based antibody drugs, it becomes quite clear that only a small segment of these proteins are dramatically different from one another, being the Fv segments that are involved in antigen binding. It should be pointed out, however, that there are some minor differences in conserved regions as well. A priori, one might assume that by finding a stable formulation for one of these antibody drugs, that such a formulation would be good for most if not all, similar antibodies. If this were borne out by experience, there would be no need for a review such as this. Instead, each antibody seems to have a unique personality related to its requirements for stability; a phenomenon derived from the fact that the small differences between these antibodies are focused on surface-exposed amino acid differences that stipulate antigen specificity. Thus, the interfacial surface of each antibody drug is unique and thus requires specific formulation components to provide maximal stability and retention of activity.

Probably the most important aspect of identifying a successful antibody drug formulation is in the identification of truly detrimental alterations rather than all of the modifications that might occur. Although, from a regulatory standpoint, it may be beneficial to understand all of the potential degradation events associated with an antibody formulation, it is the recognition of a stability-indicating parameter (or several parameters) that allows the formulation scientist to focus in on what is critical to development of a successful product. Here, a series of physicochemical and bioanalytical assays can be used to identify these parameters [32]. Although in vivo bioassays may ultimately be required to validate a stability-indicating formulation parameter, the most critical tools at the disposal of the formulation scientist are rapid and reproducible assays to measure protein changes due to oxidation, deamidation, aggregation, fragmentation and other forms of chemical modification that could lead to a loss of function of the antibody drug.

4.1. Oxidation

Methionine and cysteine residues are frequently a site of oxidation in protein drugs and this is also the case for antibody drugs [33]. In the case of antibody-based drugs, specific methionine residues within the Fc domain may be prone to oxidation, resulting in the

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production of methionine sulfoxide [25]. While cysteines are present in the Fc framework as disulfide pairs, unpaired cysteines in the variable region may also be sites of oxidation. Besides methionine and cysteine residues, oxidation of histidine, tyrosine, tryptophan and phenylalanine residues can also occur [34]. A number of methods can be used to monitor oxidation of amino acid residues. As a starting point, the number of reactive protein thiols can be monitored using reagents such as 5,5′-dithionitrobenzoic acid or iodoacethyl dansylcadaverine [35], providing a simple readout on the number of protein thiols per protein. Total amino acid analysis can also be used to monitor reductions in methionine, tyrosine and phenylalanine residues. Tryptic digestion and peptide separation by liquid chromatography followed by mass spectroscopy analysis (LC-MS) can be used to identify specific sites of amino acid oxidation.

A careful study of methionine oxidation has been performed for the humanized monoclonal antibody that recognizes the her2/neu gene product. For this protein it was determined that a methionine at position 255 in at heavy chain of the Fc region was the primary site of oxidation and that a methionine at position 431 could also oxidize under more aggressive conditions. In this study, a chromatography assay was used to detect oxidation events following papain digestion to separate Fab and Fc segments of the antibody [36]. Another study on the stability of this same protein provides a valuable template for dealing with antibody oxidation: identification of a critical oxidation event, validation of a robust assay for monitoring that event and comparison of formulation parameters to determine conditions that facilitate oxidation. Addition of antioxidants or stoichiometric levels of free methionine or thiosulfate were found to reduce the extent of oxidation in formulations of this antibody [37].

Issues associated with administration of oxidized forms of an antibody may extend beyond concerns of reduced efficacy. Systemic lupus erythematosus (SLE) is a disease characterized by the induction of anti-double-stranded DNA antibodies. Studies have shown that increased levels of markers of protein oxidation, including those of methionine sulfoxide and 3-nitrotyrosine, correlated with SLE disease activity [38]. Additionally, oxidative modifications of antibodies isolated from synovial fluid samples collected from patients with rheumatoid arthritis suggested alteration of histidine, methionine, tyrosine and cysteine residues [39]. While no direct causal association is shown or implied, these observations bring attention to the issue that we do not fully understand the clinical impact of antibody oxidation events.

4.2. Deamidation

Glutamine and asparagine residues show a propensity for deamidation [40]. Both light and heavy chains of antibodies can undergo deamidation of these amino acids [41] and deamidation events are considered to be one of the major sources of charge heterogeneity of monoclonal antibodies [42]. Deamidation events appear to be highly selective events for individual antibodies. For example, analysis of several monoclonal antibodies that bind the her2/neu gene product demonstrated asparagine 30 deamidation on one or both light chains and asparagine 55 on the heavy chain [43]. Deamidation events lead to more acidic forms of the antibody through the acquisition of additional carboxylic acid groups. Conversely, aspartic acid residues can also undergo modification to a succinimide moiety that results in a basic antibody isoform of the antibody by removal of a carboxylic acid group [43]. In general, deamidation events appear to be relatively slow but are accelerated by unfolding of the antibody [44]. Initial detection of deamidation in antibody preparations is typically identified by differences in charge distribution or content using methods such as isoelectric focusing (IEF) or high-performance cation-exchange chromatography [43].

High performance chromatography can then be used to separate succinimide, isoaspartic and aspartic acid isoforms of tryptic digest fragments of the antibody which can be unambiguously characterized by tandem mass spectroscopy (MS). Correlation between tryptic digest tandem MS results and some other more readily accessible method, such as IEF, can provide a rapid format to follow antibody deamidation events. Using these techniques, asparagine deamidation events residues appears to occur more frequently than glutamine deamidation and specific asparagine residues appear to be selectively targeted for this event [33]. Deamidation of asparagine is frequently associated with rearrangement

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events that lead to the formation of isoaspartic acid [42] and this outcome is correlated to amino acids adjacent to the asparagine prone for deamidation [44] that provide a method for prediction of potential deamidation sites (BOX). In particular, asparagine–glycine sequences can demonstrate accelerated deamidation rates since glycine residues lack a side-chain structure that would otherwise act to decreased conformation interference for backbone-associated isoaspartyl formation [45].

Box 1

Amino acid* sequences predicted to deamidate
SNG, LNG, LNN and ENN
(Note these have a following amino acid of G or N)
and those that are less prone to deamidation
GNT, GNS, GNV, TNY, SNY, SNF, SNT, SNK, SNL,
WNS, WNS, FNW, FNS, FNR HNA, HNH, LNW,
CNV, NNF, DNA, and YNP
*standard single amino acid letter nomenclature.

Non-enzymatic parameters of asparagine and glutamine deamidation in proteins have been defined in detail for factors such as pH, temperature and ionic strength [46,47]. The primary sequence dependence of these deamidation events can be explained by a simple steric and catalytic model [46,47]. Prediction of deamidation events based upon three-dimensional structures have also been described but the data set for these studies is not yet sufficient to produce a general model [48]. That asparagine and glutamine deamidation appear to be a constant phenomenon of proteins and correlate with their turnover provides the basis for the hypothesis that the presence and distribution of these amino acids within a protein can act as a molecular clock for its biological turnover [49,50]. For the formulation scientist, empiric assessment of factors such as pH and ionic strength provide the best strategy to identify a formulation that minimizes deamidation events. In general, deamidation does not result in a decrease in potency and deamidation may be hard to detect as the charge difference is small. At times, however, there can be a detrimental effect on potency through the introduction of an unfavorable negative charge that can have further ramification on biological function, particularly when deamidation occurs in the binding regions [51]. As particularly reactive residues are identified, it may be possible to have the antibody engineered to remove the problematic amino acid or to change adjacent residues to reduce its reactivity.

4.3. Aggregation

Liquid formulations of protein therapeutics frequently exhibit a decrease in concentration due to adsorptivity to container walls at low concentration and can show aggregation events at high concentrations. Initial preparations of antibody drugs were administered in acute situations by IV infusion that could allow for medium protein concentration (1–10 mg/ml) formulations not dramatically affected by adherence or aggregation losses. As antibody-based drugs became more widely used in chronic care, high concentration formulations that would allow for clinically acceptable SC injection volumes became desirable and aggregation became an important concern for designing formulations. Along with aggregation events, protein solutions at concentrations >50 mg/ml may become sufficiently viscous as to no longer readily pass through the gauge of a needle that would be comfortable for patients' use. Some antibodies demonstrate high concentration viscosity issues while others do not. For example, the anti-respiratory syncytial viral antibody Synagis® is formulated at 100 mg/ml protein and is administered successfully by IM injection. Thus, addressing viscosity, adsorptive loss and aggregation in any specific antibody formulation will likely be performed on a case-by-case basis.

As protein–protein contact frequency increases at high concentrations, the opportunity for stable aggregate formation increases proportionally. Proteins are folded in such a way as to internalize hydrophobic domains and surface-expose more hydrophilic domains in a thermodynamically stable situation. As folded proteins undergo normal molecular motion transient surface exposure of hydrophobic domains can lead to protein–protein contacts that hide these

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surfaces in a more energetically favorable manner. The outcome of these events is the stabilization of these protein–protein contacts with the ultimate outcome of dimerization, trimerization and so on — leading to extended aggregation events [52]. As protein concentration and temperature goes up, so does the probability of energetically favorable contacts leading to aggregation with increased frequency and duration of reversible protein–protein contacts leading to a greater extent of stable (even irreversible) aggregation events. Aggregation (covalent, non-covalent, dissociable, non-dissociable) must be considered a likely degradation event for high concentration antibody formulations.

For some antibodies reduced temperatures can incite reversible self-associations; these molecules are known as cryoimmunoglobulins [53]. IgG3 cryoimmunoglobulins have been shown to self-associate through sugar structures of the Fc domain [54]. Self-association events involving IgG1 antibodies seem to occur primarily through weak ionic and hydrophobic interactions [55]. More recent studies have examined kinetic and thermodynamic aspects of antibody solution dimerization using a recombinant human monoclonal antibody that recognizes vascular endothelial growth factor (rhMab-VEGF) as a model [56]. It was found that for this IgG1 class antibody, aggregation rates were greater in slightly alkaline (pH 7.5–8.5) compared to slightly acidic (pH 6.5–7.5) conditions. A high salt environment (1 M NaCl) also enhanced dimerization. Not surprisingly, self-association events were accelerated as the storage temperature was increased. One of the most striking aspects of these dimerization studies on rhMab-VEGF is that trastuzumab, another genetically engineered antibody with the same IgG1 framework and a 92% sequence homology with rhMab-VEGF, fails to demonstrate aggregation events under the same conditions.

A number of additives have been identified that can reduce the rate of protein aggregation [52,57]. A rather wide spectrum of agents can reduce protein aggregation rates: urea, guanidinium chloride, amino acids (in particular glycine and arginine), various sugars, polyalcohols, polymers (including polyethylene glycol and dextrans), surfactants and even antibodies themselves. Studies have shown that different proteins benefit from different types of anti-aggregating factors, consistent with the notion that the interfacial surfaces that drive interactions leading to aggregation are protein-specific. Conceptually, anti-aggregating agents might fall into one of two categories: small agents that fit into channels or grooves and large agents that might interact with lower-curvature surfaces of the protein. In essence, the small agents would act to stabilize a protein from acquiring a conformation that might be more reactive toward aggregation while a large agent might act to reduce the frequency of surface contacts that lead to an aggregation event. In the case of antibody formulation, either or both may be important. As we will discuss, agents found to effectively protect an antibody from aggregation may differ for various stress conditions; antibodies in solution that are undergoing agitation and thermal stress may differ from those of the same antibody as it undergoes lyophilization and reconstitution.

Protection from aggregation can be limited in antibody formulations by the addition of surface-active agents such as detergents, both charged and uncharged. As an interesting aside, such surface-active agents can help reduce protein loss due to adherence to surfaces that can occur with low concentration protein formulations. Polysorbate 20 and 80 (now available from a vegetal source) are examples of detergents that are widely used to reduce the rate of protein aggregation in high concentration antibody drug formulations. A precautionary note must be sounded, however, as detergents perform this function optimally at low concentrations (0.01% to 0.05%) and can act detrimentally to participate in protein unfolding at higher concentrations, e.g., 1%.

That antibody aggregation can be accelerated by several physical conditions provides the potential for multiple methods to determine if a particular antibody drug is prone to aggregation and to rapidly test formulations for their ability to impede aggregation. Agitation studies are done, in addition to thermal stability studies, to determine if aggregation can occur in a given formulation. Different test methods are used, employing a shaker or a stirring apparatus. In one study done on an IgG1 antibody found sizes of aggregates produced by the two methods were comparable [58]. They concluded that a higher throughput test could be constructed using stirring analyzed by absorbance measurement. In their work,

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the stirred samples exhibited much higher absorbance and second particle species in DLS, indicating that stirring stress produces a higher amount of smaller protein aggregates. While Mahler found polysorbate 80 protects against aggregation, the stirred samples had an increase in turbidity and more aggregate products were detected by DLS as compared to a surfactant-free formulation. Polysorbate 80 stabilizes small aggregates and prevents further progress in the aggregation process.

4.4. Fragmentation

Intact, full-length antibodies are composed of several segments that have natural flexure sites. One site, in particular, exists at the flexure point between the Fc and Fv domain. Cleavage at this hinge region is a common concern when assessing antibody drug stability [25]. Molecular sizing methods such as sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and size-exclusion high performance liquid chromatography (SE-HPLC) can be used to rapidly identify fragmentation events in antibody drugs [59]. Fragmentation of full-length antibody drugs is a common occurrence and invariably affects their function. In general, cleaved forms are present in such low amounts that it is likely that little change in efficacy would be seen [60]. At the very least, the antibody drug may have some small reduction in efficacy following fragmentation or may be more prone to proteases, degradation and clearance events that would limit the duration of its efficacy. At the worst, a fragmented antibody preparation may result in a different biodistribution and thus a different safety profile. This can be of particular concern if the antibody is being used to delivery a cytotoxic chemotherapeutic agent. Fortunately, the extent of fragmentation in an antibody can be easily limited in the formulated product.

5. Strategies to stabilize antibody formulations

While liquid antibody formulations are less expensive, faster to develop and generally easier to prepare for administration than alternative formulation approaches, liquid antibody formulations are prone to oxidation, deamidation, aggregation and fragmentation as discussed above. In each of these events, water is the common culprit: water mediates electron transfer during oxidation and deamidation events and its required addition across a peptide bond can be a critical step in fragmentation. The thermodynamic stresses that lead to protein aggregation are caused by hydrophobic protein surfaces being exposed to water and trying to find a lower energy state by non-native protein–protein interactions. Thus, water is a big problem for stabilizing antibody-based drugs. Lyophilization or introduction into hydrophobic polymer systems can reduce the impact of water on antibody drug formulations.

5.1. Lyophilization

Although some antibodies have demonstrated a propensity to undergo precipitation as they are cooled from 37 °C [53], antibodies are typically robust to freeze/thaw cycles [61]. Early studies reported that antibodies were stable to the freeze-drying (lyophilization) process in the absence of excipients that function as cryoprotectants [62], however, with the technical advancement of certain analytical methodologies, became evident that antibodies are generally damaged in some way as a result of lyophilization as demonstrated by extensive formation of insoluble aggregates [61]. More recently, studies have shown that freeze-drying antibodies to a specific percent of residual water (usually between 1 and 8%) allows for optimal stabilization in the dry state and stability during reconstitution [63]. Higher moisture content in lyophilized antibody preparations correlates with increased aggregation (as seen by SEC) and asparagine deamidation and isomerization (as measured by HIC). Studies have also been performed to identify optimized lyophilization schedules where the secondary drying phase is performed at an elevated temperature [64]. This technique allows the lyophylized cake to achieve optimum moisture content, allowing the final product to be conformationally stable (as measured by DSC) during extended storage. Solid-state structural studies can be used to monitor conformational changes and aggregation events during the storage of a lyophilized monoclonal antibody preparation [65]. Improved native-like structure and a reduction in aggregation can be obtained by incorporation of a carbohydrate excipient in sufficient

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quantities to fulfill the hydrogen bonding requirements on the protein surface, suggesting a critical role for non-water molecules to act as placeholders for water molecules during drying and in the dry state. That the addition of excess carbohydrate failed to improve the stabilizing function of this excipient, reaffirms the conclusion that antibodies have a limited number of sites that must be protected in this fashion [65]. A variety of carbohydrates or polyol compounds such as sucrose, trehalose and mannitol have been shown to provide this stabilizing function [66,67].

The rate of rehydration when water is reintroduced during reconstitution of lyophilized formulations is a critical parameter. As water is removed during the different drying stages of the lyophilization cycle and replaced by a shell of non-water excipients, the antibody drug can undergo structural modifications that result in a non-native conformation as may be seen by differential scanning calorimetry [63]. If the rate of rehydration is sufficiently slow to allow recovery of native conformation as water replaces non-water excipient molecules, reconstitution will typically provide a satisfactory outcome for the antibody. If, however, the antibody does not have sufficient time or capacity to recover its native state during reconstitution, extensive aggregation can result. Efforts to protect monoclonal antibodies from the rigors of freeze-drying and reconstitution have been aided by a number of analytical methods that can examine protein modifications. In the solid state, circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopy may be used to evaluate the protein; after reconstitution in the liquid phase, SEC or SDS-PAGE have been utilized to gather information from lyophilization studies [68,69]. A microscopic apparatus to visually monitor freeze-drying events has even been described [70]. Excipients that have been reported to slow the reconstitution rate and thereby preventing such changes in the rehydrated antibody are glycerol [71,72] and polymeric materials such as polyethylene glycol [73].

Pharmaceutical scientists frequently use increased temperatures for accelerated formulation stability studies. Although such studies can be used to assess formulation parameters such as pH and ionic strength, antibodies lyophilized in the presence of a cryoprotective sugar excipient provide a more complicated picture. Lyophilized amorphous solids composed of a monoclonal antibody and a sugar cryoprotectant material such as sucrose or trehalose form glassy structures, liquids that are too viscous to flow. That antibody-excipient complexes exhibit a glass transition temperature (Tg) provides the basis for observations showing that these materials have unique properties above and below this value. Thus, in predicting shelf life in accelerated degradation studies, it is important to use testing temperatures that do not exceed the Tg threshold of the complex [67]. In general, lyophilized formulations containing trehalose may likely show less tendency toward antibody aggregation than those with sucrose, since trehalose preparations have a higher Tg [67]. With this in mind, a promising approach to assess secondary structural parameters in evaluating antibody formulation stability is the use of amide I band Raman spectroscopy. This method demonstrates a good correlation between structural protein perturbations immediately after lyophilization and the rate of aggregation during long-term storage under accelerated conditions [74].

5.2. Polymer delivery systems

Removal of water from antibody preparations, either by lyophilization or spray freeze-drying, provides a provides a starting point for incorporation of this dried material into a hydrophobic polymer matrix such as polylactide-co-glycolide (PLGA) that can be made into microspheres generated through a solid-in-oil-in-water (S/O/W) encapsulation process [75]. Although both mannitol and trehalose can stabilize human IgG during spray freeze-drying, the double-emulsion solvent evaporation method used to load spray freeze-dried human IgG into PLGA microspheres has been shown to produce extensive antibody aggregation [75]. Such an outcome is likely the result of thermodynamic stress experienced at the water/organic solvent interface and a variety of process methods and excipients can be use to minimize this concern [76].

One feature of PLGA microparticle preparations is their recognition by immune elements and their capacity to stimulate immunization against incorporated proteins [77–79]. While this has clear advantages for vaccines, it poses a serious concern for therapeutic protein delivery. Hydrophobic microparticles have been reported to incite local inflammation

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at subcutaneous injection sites [80], events that may be partially mediated through macrophage recruitment [81]. Not only is the potential for antibody formation increased with local inflammation, the resulting injection site reaction may be clinically unacceptable.

In spite of some serious caveats, polymeric microspheres and delivery systems can be successful antibody formulations if administered to the appropriate sites, using certain types of polymers or preparing microspheres or polymeric formulations by methods that are more protective of the antibody. An example of a certain delivery site that may have less proclivity towards an inflammatory response is reported, where PLGA microspheres loaded with an anti-VEGF antibody were injected into the intravitreal humor of the eye, an immune-privileged site, for the treatment of age related macular degeneration (AMD) [82]. Resorbable polymers that show less potential to incite inflammation, e.g., have smoother surface characteristics, might be used to deliver antibodies in a sustained manner. Direct, local delivery of antibodies using carboxymethylcellulose aqueous gels can be an effective post-surgical anti-infective strategy [83]. Antibodies covalently coupled to a biodegradable hyaluronic acid hydrogel through a labile hydrazone linkage have been used for the sustained delivery of antibodies to sites within the central nervous system [84]. Introduction of dry powder IgG into ethylene–vinyl acetate copolymer (EVAc) has also been examined as a potential polymer-based antibody delivery system [85]. Similar to PLGA systems, EVAc microspheres can extend the time course of IgG release. This property makes polymers loaded with antibodies interesting opportunities for injectable sustained release delivery formats but also for topical application at mucosal surfaces. For example, long-term vaginal delivery of an antibody can be achieved in a mouse model using polymer vaginal rings [86,87]. Formulations of a biomedical grade polyurethane hydrogel with an antibody coating have also been prepared and shown to release bioactive protein [88]. It is important to note that one complicating aspect of many of these approaches is the requirement of a polymer solubilization step using a solvent, such as methylene chloride or isopropanol, which can compromise protein drug stability. Sustained release formulation approaches such as microspheres prepared using water, such as the PROMAXX microspheres (Epic Therapeutics) or crystallization of proteins [89] can bypass the use of solvents that are incompatible with proteins and antibodies.

6. Formulation issues for alternative forms of antibody delivery

To date, antibody development has focused primarily on injectable routes of administration: intravenous (IV), intramuscular (IM) and subcutaneous (SC). A long history of IV antibody infusions exists [90] and the advantages are clear for antibody-based drugs that are used in cancer treatments in order to reach tumor sites that may be diffuse or not otherwise accessible. In fact, the majority of the approved antibody-based drugs are administered by IV infusion (Table 1) with the total dose administered approaching one gram of protein per treatment in some cases. For many antibody-based therapies a critical safety concern involves the onset of acute or delayed systemic reactions following infusion [91,92]. Parameters and characteristics of the "infusion reaction" as well as treatment protocols have been well described with acute events commonly occurring within 10 min to 4 h and delayed events occurring 24 h to 4 days post dosing [93]. Although formation of antibodies to the administered antibody drug appear to be a risk factor for infusion reaction events [94], infusion reactions can occur following the initial exposure [93] and these reactions do not appear to be due to IgE responses or mast cell activation [95]. Most importantly for the patient, there appear to be treatment protocols to provide re-treatment of patients even after an infusion reaction event [93] with concomitant administration of immunomodulation molecules [94]. There are many unanswered questions about the mechanism and prevention of adverse infusion reaction events associated with IV infusion of antibody drugs. Certainly, one method is to explore IM and SC delivery when possible, although these delivery approaches may also have confounding aspects of tissue response and volume limitations.

Other routes are also amenable to antibody delivery. The increased number of antibody therapeutics on the market and the greater exploration of non-life threatening indications being treated by antibodies, drive efforts to deliver these agents by routes other than IV

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Table 1
Formulations of approved products

Antibody product; company	Generic name; description	Delivery route	Formulation concentration	Buffer components; pH[a]	*Excipients*
Othroclone OKT3®; J & J-Ortho Biotech	Muromonab-CD3; Murine IgG2a, anti-CD3	IV	1 mg/ml	Na,K phosphate; pH 7	NaCl, polysorbate 80
Reopro®; Centocor-Lilly	Abciximab; Chimeric IgG1, anti-GPIIb/IIIa, Fab	IV	2 mg/ml	Na phosphate; pH 7.2	NaCl, polysorbate 80
Rituxan®; Idec-Genentech	Rituximab; Chimeric IgG1, anti-CD20	IV	10 mg/ml	Na citrate; pH 6.5	NaCl, polysorbate 80
Zenapax®; Roche	Daclizimab; Humanized IgG1, anti-CD25	IV	5 mg/ml	Na phosphate; pH 6.9	NaCl, polysorbate 80
Simulect; Novartis[b]	Basilixamab; Chimeric IgG, anti-CD25	IV	4 mg/ml	Na,K phosphate, glycine	NaCl, sucrose, mannitol, polysorbate 80
Synagis®; MedImmune[b]	Palivizumab; Humanized IgG1, anti-RSV	IM	100 mg/ml	Histidine, glycine	Mannitol
Remicade®; Centocor[b]	Infliximab; Chimeric IgG1, anti-TNFα	IV	10 mg/ml	Na phosphate; pH 7.2	Sucrose, polysorbate 80
Herceptin®; Genectech[b]	Trastuzumab; Humanized IgG1, anti-HER2	IV	21 mg/ml	Histidine; pH 6	Trehalose, polysorbate 20, benzyl alcohol[c]
Mylotarg®; Wyeth[b]	Gemtuzumab ozogamicin; Humanized IgG4, anti-CD33, immunotoxin	IV	4 mg/ml	Na phosphate	NaCl, sucrose, dextran 40
Campath®-1H; Millennium-ILEX	Alemtuzumab; Humanized IgG1, anti-CD52	IV	Unclear — 30 mg /vial	Na,K-phosphate pH 7	NaCl, KCl, NaEDTA, polysorbate 80
Zevalin®; Biogen Idec	Ibritumomab tiuxetan; Murine IgG1, anti-CD20; radiolabeled (^{90}Y or ^{111}In)	IV[d]	1.6 mg/ml	Na Acetate, Na, K-phosphate pH 7	NaCl, human albumin
Humira®; Abbott	Adalimumab; Human IgG1, anti-TNFα	IV	50 mg/ml	Na phosphate, Na Citrate; pH 5.2	Mannitol, polysorbate 80
Xolair®; Genentech[b]	Omalizumab; Humanized IgG1, anti-IgE	SC	125 mg/ml	Histidine	Sucrose, polysorbate 20
Bexxar®; Corixam-GSK	Tositumomab-I131; Murine IgG2a, anti-CD20, radiolabeled (^{131}I)	IV	14 mg/ml	Na phosphate; pH 7.2	NaCl, maltose
Raptiva®; Genentech	Efalizumab; Humanized IgG1, anti-CD11a	SC	100 mg/ml	Histidine; pH 6.2	Sucrose, polysorbate 20
Erbitux®; Imclone Systems	Cetuximab; Chimeric IgG1, anti-EGFR	IV	2 mg/ml	Na phosphate; pH 7.2	NaCl
Avasatin®; Genentech	Bevacizumab; Humanized IgG1, anti-VEGF	IV	25 mg/ml	Na phosphate; pH 6.4	Trehalose, polysorbate 20
Tysabri; Biogen Idec	Natalizumab; Humanized IgG4, anti-4α-integrin	IV	20 g/ml	Na phosphate; pH 6.1	NaCl; polysorbate 80

Ordered by approval dates in the US.
Abbreviations: RSV=respiratory syncytial virus; TNFα=tumor necrosis factor alpha; EGFR=epidermal growth factor receptor; VEGF=vascular endothelial growth factor; NaEDTA=sodium edentate.
[a] Some lyophilized formulations do not stipulate a pH (or range) following reconstitution.
[b] Lyophlized product, concentration, pH, etc. values are obtained after reconstitution.
[c] Added as a preservative to generate a multi-dose vial configuration.
[d] Kit where components are mixed just prior to administation.

infusion. These routes may be more effective, less invasive, provide easier delivery, permit self-administration and thereby be more convenient and possibly have lower costs if less active agent can be used, as in the case where the alternate route of administration delivers the therapeutic agent to the intended site directly. Interestingly, some studies have suggested that antibodies administered by IV infusion are more

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susceptible to proteolysis than via IM injection [59] and sucrose-stabilized immunoglobulins have been associated with acute renal failure following IV infusion [96]. The antibody used to protect neonates from respiratory syncytial viral (RSV) infection (Synagis®) is one example of an antibody administered by IM injection. For reasons discussed above, the majority of antibody-based drugs have moved toward SC delivery with formulations efforts being focused to this end. Based upon the desired actions for a number of antibody-based drugs, however, routes of administration other than IV, IM or SC may be desirable. These alternative routes can provide unique formulation and stability issues.

6.1. Oral

Uptake of antibodies from the intestinal lumen can occur through fetal Fc receptors (FcRn) expressed at the apical surface of enterocytes [97]. This natural antibody uptake mechanism was initially identified in the gastrointestinal (GI) tract of neonatal rodents and its expression shown to vanish following "closure" of the intestine after weaning. It was only recently that the continued expression of this receptor in the adult intestine was demonstrated and this finding has prompted efforts to examine this receptor system as a means of delivery antibodies after oral administration. Although this pathway appears to be a viable route for the delivery of therapeutic antibodies, issues associated with antibody stability in the GI tract [98] and competition from endogenous antibodies can dramatically reduce the efficiency of uptake after oral delivery. Outside of this specific mechanism, no other transcellular mechanism of efficient antibody transport across epithelial cell of the GI tract has been demonstrated to date. Topical application of antibodies to produce a desired clinical outcome have been shown, however, with the oral delivery of a chicken-derived (IgY) antibodies for treating and preventing infectious diseases of the GI tract [98–100]. It should be noted that IgY antibodies have been shown to not bind to (or be transported by) FcRn [97].

6.2. Respiratory tract

Intranasal and pulmonary administration of protein therapeutics has shown more promising clinical outcomes than oral delivery. While the surfaces of both the respiratory tract and the gastrointestinal tracts are readily accessible, the respiratory tract is a far less hostile environment for an administered protein therapeutic. Nonetheless there are physical clearance mechanisms in place to remove foreign particles which act to reduce residence time dramatically following administration. In the case of nasal administration the use of a cream emulsion and a polymer delivery system has been shown to significantly decrease the clearance rate for a monoclonal antibody when applied to the nasal mucosa [101]. This study provides one approach for the topical application of a therapeutic antibody designed to provide clinical benefit at the mucosal surface. It does not, however, suggest that an appreciable amount of an antibody might be absorbed into the systemic circulation from this site. The nasal mucosa affords only a small absorptive surface area compared to the epithelial linings of the lung.

Aerosol delivery of antibodies to the lung has been explored as either a liquid or dry powder formulation, where in both cases the droplets or particles must be of sufficiently small size that they are respirable perhaps to the level of the deep lung but large enough that they are not readily exhaled or just impact the back of the throat [102]. The sizes of particles that provide this performance have a mass mean aerodynamic diameter and density such that they enter the alveoli and remain in the lung. This size window has been reported to be between 1 to 5 μm depending on the formulation used and in one case, far larger, 20 μm where the particles are dense (<0.4 g/cm^3) and highly porous [102].

Formulation efforts were made for producing fine dry powder particles of an anti-IgE monoclonal antibody (E25) in the size range desired for delivery to the lung [103]. These studies showed that sodium phosphate was capable of extending the usefulness of mannitol as a protective excipient in spray-dried antibody formulations and that mannitol can play a role both in maintaining protein stability as well as producing a suitable aerosol preparation. Further studies to assess carbohydrate excipients for spray-dried aerosol E25 formulations showed that excipient to protein ratios were critical. Degradation issues involved not only protein aggregation but also protein glycation or lactosylation, a protein modification

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event where a reducing sugar can add covalently at a free amine [104]. The ability to formulate and delivery an antibody-based drug to the lung provides the potential for systemic delivery of that protein via uptake through the FcRn expressed at this mucosal surface. Elegant studies showing the distribution and function as well as the application of this transport route for systemic deliver of an Fc-containing protein chimera has been demonstrated [105,106]. Thus, the potential or systemic delivery of antibody-based drugs via the respiratory tract looks promising.

6.3. Miscellaneous delivery sites of the body

Unique aspects of antibody–polymer formulations designed for intravitreal injection [82] or topical vaginal delivery [86,87] have been discussed above. Additionally, antibody formulations using hyaluronic acid hydrogels for sustained brain delivery have been noted [84]. These methods focus on strategies to administer large amounts of an antibody at a specific site in order to provide a sustained level to improve efficacy and/or to minimize injection frequency. Other strategies have been described to deliver antibodies to miscellaneous sites throughout the body, For example, an anti-transferrin antibody (OX-26) has been described as a carrier for delivery of covalently attached therapeutic molecules across the blood–brain barrier following IV injection [107]. An anti-tumor necrosis factor antibody has been adsorbed onto cardiovascular stent wires for local neutralization of this cytokine in order to limit restenosis events following stent placement [108]. A CMC gel containing antibodies to prevent post-surgical infections has been administered to the intraperitoneal cavity [83].

6.4. Intracellular targeting

Some promising antibody drug targets are intracellular. Although techniques exist where a cell can synthesize an antibody and secrete it into the cell cytoplasm, successful use of this technology requires gene therapy. Instead, it is possible to chemically couple an antibody to a pH-sensitive polymer that functioned to disrupt membranes at acidic pH. Many antibodies are internalized by cells through a receptor-mediated uptake process that leads to their delivery into early and then late endosomes. During endosomal maturation after receptor–antibody internalization the pH of these vesicles will drop from near neutral to below pH 6. This approach was demonstrated in a format where the pH-responsive polymer poly(propylacrylic acid) was covalently attached to a monoclonal antibody to facilitate its delivery to the cytoplasm of target cells [12].

7. Other delivery issues

A wide range of novel drugs and drug conjugates are being developed using monoclonal antibodies and engineered antibody fragments [20]. For some of these novel therapeutic approaches to be successful, they require additional or non-traditional delivery strategies. A cursory discussion of some of these approaches as they relate to intracellular targeting, site placement and engineered antibodies fragments follows.

7.1. Antibody fragments

An entire spectrum of antibody fragments have been identified through protein engineering studies with many of these showing remarkable potential for unique and novel clinical opportunities [109]. These smaller recombinant antibody fragments, the monovalent antibody fragments, Fab and single chain Fv and the engineered variants, diabodies, triabodies, minibodies and single-domain antibodies are an emerging form of antibody therapy that are less costly to manufacture while still retaining the targeting specificity of full length antibodies. In fact some may have greater efficacy and more applications than whole monoclonal antibodies. It may be anticipated that this new subset of antibodies are posing new formulation challenges, however, to date, publications detailing stability studies and formulation development for these molecules are rare in the scientific literature so formulation data on antibody fragments is scant. The single V domains have been found to be poorly soluble and have a tendency to aggregate [109], but these problems are being overcome by the identification of mutants that minimize the hydrophobic interface and selected by phage display [110]. Five Fab molecules have been approved as of this writing with the one humanized

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Fab (ranibizumab rhFab) awaiting approval in the very near future, but many are in early clinical trials and most in preclinical development. One study has been described that focused on Fab fragments. In general, the binding of an antibody to an effector protein does not necessarily inactivate that target's function [111]. When an antibody is effectively inactivating, however, the Fab fragments in the absence of an Fc domain can frequently function as highly potent binding agents capable of masking binding sites or enzymatic elements on macromolecules. For example, Fab fragments prepared from sheep anti-snake venom antibodies (Digibind, from GSK) can be an effective anti-venom. Storage of liquid formulations of such anti-venoms in sub-tropical countries where refrigeration may not be possible provides a significant formulation challenge. Although lyophilized products can perform well under these conditions, a pH 4.0 (acetate buffer) formulation was found to also be stable and potent for at least one year at room temperature [112], presumably room temperatures characteristic of the tropical and subtropical regions where the material would be used. Strikingly, this stable formulation did not require the presence of a protective carbohydrate excipient. To improve drug delivery, antibody fragments have been fused to many types of molecules, such as radionuclides, cytotoxic drugs, toxins, peptides and proteins, enzymes and liposomes [113]. An immunoliposome made with a Mab fragment is reported to deliver drugs to the brain as such a molecule is able to cross the formidable barrier of the blood brain barrier [114].

7.2. *Immunoconjugates*

Arming antibodies with materials useful for detection or cell killing has become a prominent approach to increase the utility of these proteins, reviewed by Wu and Senter, [20]. Antibodies provide a useful format for the production of radiopharmaceuticals for site- or tissue-specific localization using gamma scintigraphy [10,115] or for enhanced cell killing. There are currently three approved antibody–radionuclide drugs: two are murine radiolabeled Ab for B cell lymphomas ibritumomab tuixetan (^{90}Y, Zevalin) and tositumomab (^{131}I Bexxar) and the third is a humanized, gemtuzumab ozogamicin (calicheamicin, Mylotarg) for leukemia. The presence of a high energy emitting radionuclide, however, will compromise long-term stability of any antibody formulation; not only would the released energies act to damage the protein but there is strong preference to use short half-life isotopes for these procedures [116]. Thus, in these instances, a stable formulation based upon the principles discussed above can provide a product that can be radiolabeled and then used soon after this step. One example of this approach is a lyophilized antibody formulation that is reconstituted and prepared by a kit method prior to patient dosing [117].

Antibodies coupled with cytotoxins for targeted cell killing [118] are particularly useful in antibody-based cancer therapies [119]. Beyond the identification of a stable formulation for the antibody, such materials have several additional problems for the formulation scientist to solve: stability issues of the attached agent and stability of the linker used to join the attached agent to the antibody. In the case of a monoclonal antibody-vinca alkaloid conjugate, a pH range of 4.5–5.5 in a phosphate buffer showed improved solution stability compared to material stored at pH 6.5–7.4 as evidenced by antibody aggregation and hydrolysis of the chemical linker used to conjoin the alkaloid to the antibody [120]. In most cases, cytotoxic materials are coupled to antibodies using reversible chemistries to provide a release mechanism for maximal activity at the target site. Many of these antibody conjugates use a disulfide bond linkage that can be reduced by a novel redox enzyme present in endosomes and lysosomes of target cells [121]. Slightly oxidizing conditions that would provide added stability of this linker in the formulation could, over time, act to cause detrimental oxidation events in the antibody and/or the linked compound. Other conjugates use an acid-labile linker [122]. In this case, formulation pH must be restricted to either neutral or alkaline conditions. Enzymatic separation of antibody and a cytotoxic material could also be achieved by coupling the two through an amino acid linker that is recognized as a substrate by a specific protease or peptidase [123]. Formulation instability of this linker sequences can be corrected to some extent by selective changes of the amino acids used.

Thus, the challenges of immunoconjugates include maintaining both drug and antibody potency and stability. While some antibodies are efficacious by

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themselves, many are much more therapeutically active after being armed with a toxin, drug and radionuclide [124]. These changes make the resulting product more difficult to prepare, stabilize and deliver. Yet, there is tremendous enthusiasm for these approaches as they provide the promise for some companies to transform their antibody into a potentially curative drug. For this reason many antibodies are combined with a generic delivery system, such as a liposome, that can be used to delivery a variety or even multiple agents simultaneously through antibody-directed targeting [125]. Examples of such an approach include studies performed using an antibody directed against the Her2/neu antigen to delivery liposomes containing a chemotherapeutic have been described [126], antibodies used to deliver cationic liposomes for the administration of nucleic acid material for gene therapy [127] and antibodies used in the targeted delivery of modified gelatin nanoparticles [128] that can be coupled through a disulfide linkage [129].

8. Conclusions — challenges and opportunities

As antibody-based drugs come to the forefront of therapeutics derived from biotechnology, they provide a range of new and exciting opportunities to treat a number of currently unmet medical needs. Successful clinical application of these novel agents requires the development of stable formulations that can be used for specific delivery methods. Antibodies, because of their endogenous nature, have built-in features that may pose problems for stability as biotherapeutics, that function in the normal clearance of these proteins from the body [40]. Thus, despite our best efforts to prepare stable antibody-based drug formulations, the ultimate stability of these products may be limited to inherent mechanisms of degradation pre-designed for the rate of clearance optimal for their function and turnover in the body. Protein engineering has led to significant advances in improving some of these parameters and the trend would predict that additional advances will be made in the near future (reviewed in [109]). Protein modifications can be engineered such that stability and efficacy can be improved in ways that cannot be achieved solely, if at all, with formulation alterations. While it is clear that more research is needed in the relatively new area of engineered antibody fragments for clinical use [109], they have great potential for diagnostic and therapeutic application.

In general, successful antibody formulation designs are simply the best compromise of minimizing several competing degradation pathways. One approach is to find a pH where the minimal amount of degradation events such as deamidation and oxidation will occur. Another is to identify a buffer system that can stabilize the formulation at that pH without damaging the antibody. One common tactic taken to reduce the rate of many of these chemical degradation events is to prepare lyophilized or freeze-dried antibody preparation but even these can be compromised by aggregation issues due to thermodynamic stress that occurs during freezing, drying and reconstitution. A number of protective excipients, mostly carbohydrates, can reduce the propensity of antibodies to aggregate during these stressful events. Another issue is to produce a formulation that is compatible with delivery — neither hypotonic — nor hypertonic solutions are well-tolerated upon injection. Added to this is the potential use of a surface active agent to help with liquid stability such as a non-ionic detergent at a low concentration [58] as well as the possible addition of preservatives required for multi-use systems [130].

The future of antibody based drugs is likely to include more complex systems than those currently approved for use in man. Possibly the most critical aspect of generating a pharmaceutically successful formulation for antibody based drugs is an understanding the critical aspects of stability for the protein or the materials associated with the protein. It has been known for some time that even a single amino acid change in the Fv region of an antibody can result in dramatic differences in antigen-binding specificity [31]. Also, alterations at specific amino acids in the Fc domain can dramatically change functional aspects of an antibody. Thus, these kinds of changes must be considered more important in the search for a successful formulation than those changes that do not appear to affect function. In the case of antibody or antibody-like agents being used to delivery a payload such as a toxin or chemotherapeutic, the stability of the material to be delivered and its continued association with the delivery antibody is crucial.

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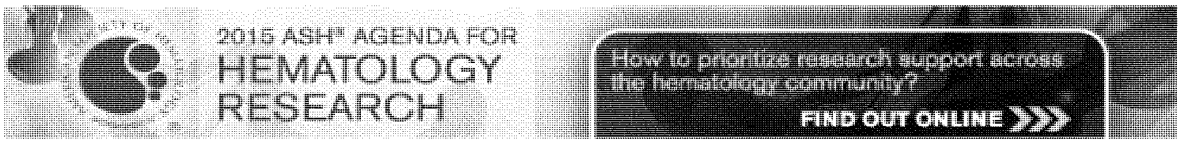
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Sustained Control of Hemolysis and Symptoms and Reduced Transfusion Requirements over a Period of 2 Years in Paroxysmal Nocturnal Hemoglobinuria (PNH) with Eculizumab Therapy.

Anita Hill, Dupe Elebute, Judith C. Marsh, Stephen J. Richards, Bruce McNutt, Beth E. Petro, Russell P. Rother and Peter Hillmen

Blood 2004 104:2823;

Abstract

Paroxysmal nocturnal hemoglobinuria (PNH) is characterized by intravascular hemolysis and venous thrombosis. Deficiency of the terminal complement inhibitor CD59 from PNH red cells results in complement-mediated hemolysis. Eculizumab, a humanized monoclonal antibody that inhibits terminal complement by binding to C5, is effective at controlling intravascular hemolysis in PNH. We now report that 10 of the 11 patients from an initial 3 month study have continued to receive 900mg eculizumab every other week for 2 years. The remaining patient stopped eculizumab after 23 months despite effective control of intravascular hemolysis, as the patient continued to be transfused even after erythropoietin therapy. This patient had the most severe hypoplasia at the start of eculizumab therapy with a platelet count below $30x10^9/l$, suggesting that the ongoing transfusions were due to the bone marrow failure and not continuing hemolysis. Eculizumab was safe and well tolerated with two reported SAEs in the last year, neither of which were attributed to the drug. The dramatic improvement in various parameters of hemolysis persisted during the 2 year treatment period for all patients. Mean LDH levels decreased from 3111 +/− 598 U/L over the 12 months prior to treatment to 634 +/− 34 U/L up to 24 months following treatment (p=0.002). PNH red cells with a complete deficiency of GPI-linked proteins (Type III red cells) progressively increased during the treatment period from a mean of 36.7% to 58.9%

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($p=0.001$) while partially deficient PNH red cells (Type II) increased from 5.3% to 8.7% ($p=0.01$). There has been no change in the proportion of PNH neutrophils in any of the patients during eculizumab therapy indicating that the increase in the proportion of PNH red cells is due to a reduction in hemolysis and transfusions rather than a change in the PNH clone(s) itself. The mean and median transfusion rates decreased from 2.1 and 1.8 units/patient/month to 0.4 and 0.3 units/patient/month respectively ($p<0.001$). Three patients remained transfusion independent during the whole 2 year treatment period and the other 7 patients remaining on eculizumab all had at least a three-fold reduction in transfusion requirements. No patients have had a thrombosis during the study period, including 5 patients who were not on warfarin therapy. Prior to eculizumab, some patients suffered with abdominal pain, dysphagia and erectile dysfunction. Following the first dose of eculizumab there has been complete and sustained resolution of these symptoms. In addition, other symptoms related to hemolysis, such as lethargy, have shown a sustained marked improvement. These classical PNH symptoms have been attributed, at lease in part, to the release of free hemoglobin (Hb) during intravascular hemolysis which overwhelms the protective Hb scavenging mechanisms. Free Hb efficiently absorbs and depletes nitric oxide (NO) which is a critical regulator of smooth muscle contraction, vascular tone and platelet activation. The potential role of NO in the symptomologies of PNH are currently under investigation. In summary, we report that eculizumab is well tolerated and has persisting efficacy as demonstrated by decreased hemolysis and transfusions, as well as improvement in the symptoms of PNH, for over 2 years of therapy.

2005, The American Society of Hematology

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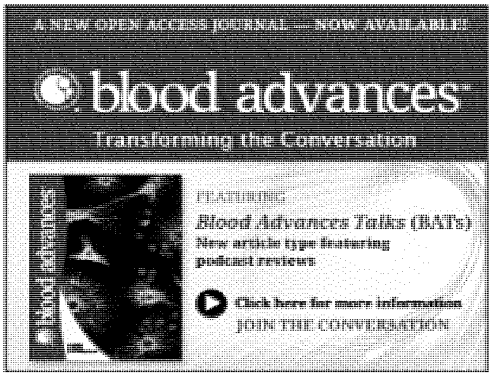
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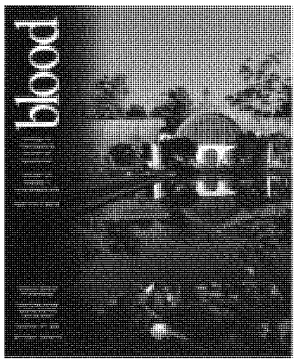


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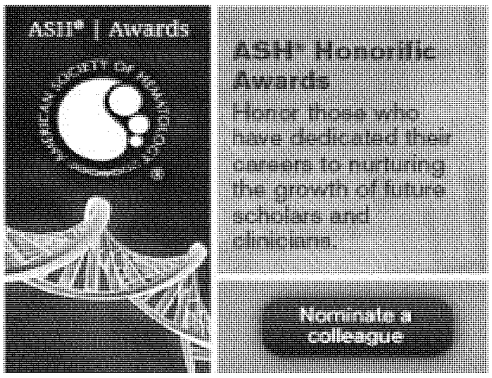
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Oral Communication

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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 *in vitro*, and these peptides inhibit T-cell proliferation in response to stimulation with purified RhD protein *in vitro*. These peptides may be used to induce tolerance by the parenteral route. We have used a HLA-DR15 transgenic mouse model to show that T-cells from mice immunized with the RhD protein proliferate in response to purified RhD protein and to RhD peptides, and serum antibodies specific for human erythrocytes are also produced. Mucosal administration of immunodominant RhD peptides, or parenteral administration of IL-10 inducing peptides prior to immunization with RhD protein, inhibits both T-cell proliferation and antibody production. These results suggest that synthetic RhD peptides can induce tolerance to the RhD protein and may be of value in the prevention of immunization in RhD-negative individuals.

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A humanized monoclonal antibody against vWF A1 domain inhibits vWF:RiCof activity and platelet adhesion in human volunteers

S. J. Machin[1], C. Clarke[2], O. Ikemura[3], S. Kageyama[4], I. J. Mackie[1], J. A. Talbot[2], Y. Ikeda[5] and Y. Gyotoku[4]

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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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high shear, it inhibits platelet adhesion, aggregation and activation, and in a dog model, inhibits arterial thrombus formation, without prolonging the bleeding time. In a randomized, double blind study, placebo, 0.01, 0.03, or 0.05 mg/kg AJW200, were infused i.v., into 24 healthy men. No clinically significant adverse events were recorded and there was no evidence of immunogenicity. Mean PK results were: C_{max} 204.8, 586.2, 833.2 ng/ml; T_{max} 1.0, 0.92, 0.57 h, T/2 23.5, 24.3, 27.2 h, for ascending doses. The maximum vWF occupancy by AJW200 was 19.4, 51.0 and 62.4%, showing correlation with plasma AJW200 concentration. Ristocetin cofactor (RiCof) was reduced 1 h post-infusion compared to baseline: mean (SD) 58 (22) and 110 (25)%, $P < 0.0005$ and 34 (16) and 116 (41)%, $P < 0.005$, at 0.03 and 0.05 mg/kg respectively, for up to 12 h. vWF : Ag, FVIII, vWF multimers and cleaving protease activity were unchanged. Template bleeding time (BT) was normal at all AJW200 doses, but the PFA-100 showed prolonged closure times 1 h post-infusion, with both CADP: 146 (22) and 82 (10) and 202 (78) and 80 (19) s, $P < 0.01$; and CEPI cartridges: 182 (28) and 108 (16) and 233 (56) and 107 (19) s, $P < 0.01$, at 0.03 and 0.05 mg/kg AJW200, respectively. This effect lasted for 3–6 h with the lower and up to 12 h for the higher dose. The RiCof and PFA-100 responses showed dose dependent inhibition and the magnitude of the effect appeared to be related to the baseline vWF level. AJW200 showed inhibition of vWF : RiCof activity and PFA-100 responses, without prolonging the skin bleeding time, and merits further clinical studies for thrombotic disorders. This is the first study demonstrating the effect of a monoclonal antibody that blocks GPIb/vWF in humans.

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Phase I study of the adoptive transfer of tetramer selected cytomegalovirus-specific cytotoxic T cells after allogeneic stem cell transplantation

M. Cobbold[1], N. Khan[1], S. Tauro[2], D. McDonald[3], E. Ollavaria[2], R. Chakraverty[2], J. Goldman[4], P. Mahendra[2], C. Craddock[2] and P. Moss[1]

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Cytomegalovirus (CMV) remains an important cause of morbidity and mortality following stem cell transplantation (SCT). Adoptive transfer of donor CMV-specific CD8+ T cell clones has been shown to protect against CMV reactivation in previous studies. However, this procedure has not been widely used due to the need for extensive *ex vivo* culture of T cells prior to infusion. We have combined HLA-peptide tetramer staining with the use of magnetic beads against phycoerythrin to facilitate the rapid selection of CMV-specific CTL from donor peripheral blood using a CliniMACS machine. In this study we infused tetramer selected donor CMV-CTL to six allogeneic stem cell transplant (SCT) recipients following CMV reactivation. An additional patient had undergone transplantation from a matched unrelated donor and CTL were infused for treatment of CMV reactivation refractory to Foscarnet, Ganciclovir and Cidofovir. In the 6 sibling allografts between 10^5 and 1.6×10^6 (mean 6×10^5) CMV-specific CD8+ T cells were infused from 250 mL of donor peripheral blood. The unrelated donor transplant patient received 2×10^6 CMV-CTL isolated from a cryopreserved lymphopheresis product. No infusion reactions were noted. One patient developed grade II GvHD which responded to treatment. CMV-CTL were not detectable prior to infusion in three of six patients but were detected in all patients following infusion. CMV-CTL expanded to represent up to 22.1% of the CD8 compartment. This expansion occurred in the first 14 days followed by a contraction to a basal level. Four patients did not require antiviral therapy. In the unrelated transplant recipient CMV-specific CTL reached a peak of 0.6% of the CD8 compartment and CMV antigenaemia became negative 6 days following CTL infusion. This novel approach to the adoptive transfer of antigen-specific CTL is rapid and relatively straightforward. It has considerable potential in the development of antigen-specific T cell therapy after allogeneic transplantation.

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Bone marrow mesenchymal stromal cells protect chronic lymphocytic leukaemia (CLL) from spontaneous and drug-induced apoptosis

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CLL cells are able to survive for prolonged periods *in vitro* and yet in most instances undergo rapid spontaneous apoptosis when cultured *in vitro*. Sustained viability *in vitro* is probably maintained by contact with 'nurse-like' cells in the peripheral blood (PB) and the bone marrow (BM) microenvironment. An *in vitro* model to mimic *in vitro* conditions was developed using adherent BM mesenchymal stromal cells (MSCs) from the iliac crest of normal donors. This model was used to test protection of CLL PB mononuclear cells both from apoptosis and from the effects of CD20 (Rituximab) and CD52 (CAMPATH-1H) therapeutic monoclonal antibodies, the proteasomal inhibitor MG132 and Bis9, an inhibitor of protein kinase C. Levels of apoptosis were determined by flow cytometry, using AnnexinV-FITC and propidium iodide. BM MSCs abrogated spontaneous apoptosis of CLL cells over a 5-day period of culture (80–90% viability, $n = 9$). Protection required CLL cells to be in close contact with the MSCs; separation by a microporous membrane induced apoptosis, comparable to that seen in control samples cultured without MSCs (30–40% viability). Rituximab treatment did not have any obvious apoptotic effect on CLL cells *in vitro* but CAMPATH-1H was able to induce apoptosis in a subpopulation of cells even in the presence of MSCs. Treatment with Bis9 and MG132 caused substantial and rapid apoptosis of CLL cells (0–15% viability $n = 3$). At lower doses of both drugs (200 nM), this apoptosis was prevented by MSC co-culture (70–90% viability). MSCs can therefore maintain CLL viability *in vitro* and can influence drug-induced apoptosis. The critical cell–cell interactions remain unknown, but are likely to include the beta-integrin, TNFSF13B and the CXCR4/CXCRL12 pathways. The expression of these molecules and their possible interactions in maintaining CLL viability will be investigated to assess if any might be targeted therapeutically.

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Targeting FGFR3 in multiple myeloma: the use of SU5402 and PD173074 to inhibit t(4;14) positive cells

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The t(4;14)(p16.3;q32), associated with 10–20% of cases of multiple myeloma (MM), deregulates the expression of two genes, MMSET and FGFR3. Most t(4;14) positive myeloma patients express FGFR3 and constitutive activation of this receptor is seen in some cases. To assess the potential of FGFR3 as a drug target we evaluated the effects of three FGFR inhibitors (SU6668, SU5402 and PD173074) on control and MM cell lines. Activity was assessed initially against Ba/F3 cells transformed to IL-3 independence by ZNF198-FGFR1, a constitutively active fusion gene associated with the 8p11 myeloproliferative syndrome (EMS), and compared to activity against control BaF3 cells grown with IL-3. Three methods were employed: MTS to assess proliferation/metabolic activity, trypan blue staining to determine cell viability and TUNEL to assess apoptosis. SU6668 inhibited the growth of both transformed and control cells lines at concentrations of >20 μM and therefore was not pursued further. SU5402 and PD173074 specifically inhibited the growth of ZNF198-FGFR1 transformed cells in a dose-dependent fashion with an IC50 of 5 μM and 5 nM, respectively. Both compounds also caused decreased cell viability and increased apoptosis. These compounds were then tested against two t(4;14) positive MM lines, KMS-11 and OPM-2, which express FGFR3 and harbour activating mutations of this gene. Dose-dependent growth inhibition was observed in both lines with IC50s similar to those in ZNF198-FGFR1 transformed cells. Importantly, inhibition was still observed in the presence of IL-6. Both compounds induced a dose-dependent reduction in cell viability and an increase in apoptosis, accompanied by a decrease in FGFR3 and ERK phosphorylation. In contrast, no inhibition was seen with either compound against the t(4;14) negative lines U266 and HS-Sultan, or the t(4;14) positive, FGFR3 negative cell line KMS-18. FGFR3 is thus a plausible candidate for targeted therapy in a subset of MM patients.

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Myeloid Malignancy

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Decreased expression of a novel growth regulatory protein, Nov, in chronic myeloid leukaemia

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Chronic myeloid leukaemia (CML) is characterized by the constitutively active Bcr-Abl tyrosine kinase. We have used a murine multipotent haematopoietic stem cell line, FDCP-MIX, expressing a temperature-sensitive mutant Bcr-Abl (ts Bcr-Abl FDCP-MIX), to investigate changes in gene expression using DNA microarray analysis. RNA was prepared from cells expressing the Bcr-Abl tyrosine kinase and from control cells, labelled with biotin and hybridized to an Affymetrix Mu6500 array (Affymetrix, USA). The Bcr-Abl kinase activity resulted in differential expression of 300 genes, the most significant being a 25-fold decrease in expression of a novel gene called nephroblastoma overexpressed (Nov). Northern blot analysis detected a 4-fold reduction in Nov expression in the Bcr-Abl positive cells in comparison with the control cells. Western blot analysis of immunoaffinity-purified Nov detected a two-fold reduction in expression as a result of the Bcr-Abl kinase activity. Confocal microscopy revealed strong vesicular cytoplasmic staining for Nov in FDCP-MIX control cells whilst fluorescence was barely detectable in cells expressing Bcr-Abl. A similar pattern of Nov expression was observed in primary human cells (five CML, five normal controls). Normal CD34+ progenitor cells uniformly expressed an intense fluorescence signal for Nov expression. In contrast, the majority of CD34+ cells within the CML patient samples were negative ($P < 0.001$). Nov expression has previously been associated with cell differentiation, quiescence and has been shown to promote cell adhesion. Loss of these properties is consistent with the CML phenotype.

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Reduced hTR expression in CML CD34+ cells at diagnosis: a novel mechanism for telomere loss in leukaemia

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CML is characterized by massive myeloid expansion, and significant telomere loss has been documented at diagnosis and during disease progression. Critical telomere shortening results in chromosomal instability, which can lead to disease progression. The regulatory mechanisms and expression levels of the key telomerase components (hTERT and hTR, the reverse transcriptase and RNA template respectively) in the CML stem cell compartment have not been described; however studies in solid tumours and cell lines generally show increased expression of both components. We selected CD34+ cells from 16 CML diagnostic leucapheresis specimens and 12 non-CML mobilized PBSC (control population) (median purity 94.1, 96.3% respectively; BCR-ABL positive in CML group). High-resolution cell cycle analysis demonstrated increased cycling in the CML CD34+ fraction. Q-RT-PCR for full-length (functional) hTERT showed an approximately two-fold rise in CML expression compared with control cells. RT-PCR with primers designed to detect the full-length hTERT transcript plus the three non-functional splice variants (with quantitative analysis) demonstrated a shift away from splicing in the CML samples. By combining these two complementary techniques we determined that expression of all (i.e. full length plus splice variants) transcripts are not different between CML and control cells: the increased levels of functional hTERT mRNA appear to be accounted for mainly by a reduction in the splicing out of the active sites. Surprisingly, Q-RT-PCR for hTR demonstrated a five-fold reduction in hTR levels in CML compared with control cells. Even more striking was the difference in hTERT/hTR ratio: 1 : 180 in CML cells versus 1 : 1800 in non-CML cells. We therefore hypothesize that despite increased hTERT levels there is a disassociated reduction in hTR, which in conjunction with increased cell cycling and replication

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induced telomere loss, could alter telomere homeostasis in CML. This could contribute to disease evolution via chromosomal instability.

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Reduced-intensity allogeneic haematopoietic stem cell transplantation (HSCT) using fludarabine, busulphan and alemtuzumab conditioning in 63 patients with myelodysplastic syndromes (MDS)

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We report the largest series of patients with MDS who have received a reduced-intensity HSCT using Fludarabine 30 mg/m^2 i.v. (day −9 to −5); busulphan 4 mg/kg/day orally (day −3 to −2) and alemtuzumab 20 mg i.v. (day −5 to −1). Immunosuppression comprised ciclosporin from day −1 titrated to plasma troughs of 150–200 ng/L. 63 patients with MDS (RA = 16, RAEB-I = 9, RAEB-II = 11, CMML = 5, TLD-AML = 21, aCML = 1) have received transplants from matched sibling (MSD) (24) or volunteer unrelated donors (VUD) (39). Except for RA, all had received at least one course of chemotherapy. Twenty-two of 24 MSD utilized PBSCs compared with 19 of 39 VUD HSCTs, with a median CD34+ cells of 4.6×10^6/kg (1.83–17.5) and 3.9×10^6/kg (0.44–16.7) respectively. There was no difference in engraftment latency, with the median time to neutrophil engraftment of 15 days (4–57) and platelets 18 days (7–105). 85% achieved full donor chimerism with a 6% incidence of grade III/IV aGvHD (43% grade I–IV) and 3% extensive cGvHD. At a median follow-up of 343 days (50–1213) for MSD, and 248 days (37–1316) for VUD recipients, 63 and 62% were alive respectively. The median age of sibling allografts recipients was 54 years (41–70). At day +200, the TRM was 5% and DFS 68%. The median age for VUD recipients was 49 years (25–58). The TRM at day +200 was 21% with DFS 65%. At 1 year, the estimated OS was 66% for VUD and 77% for MSD, with a DFS of 57 and 53% respectively. Twenty-one received DLI at a median of day +273 (126–1290); 11 for relapse and 10 for declining donor chimerism. A graft versus leukaemia effect was demonstrable. Although longer follow-up is required, RIC allogeneic HSCT appears to be safe and feasible in this high-risk group of patients.

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Combined clofibric acid and medroxyprogesterone acetate exert multiple anti-leukaemic actions against acute myeloid leukaemia cells and cell lines

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Poor survival rates in acute myeloid leukaemia (AML) relate to both the failure of cytotoxic chemotherapy and inability of older patients to tolerate that therapy. Thus non-toxic drugs that specifically target the differentiation and apoptotic pathways of AML cells are urgently required. We and others have demonstrated that fibrates such as clofibric acid (CA) and inhibitors of the aldo-keto reductase AKR1C3, including medroxyprogesterone acetate (MPA), potentiate all trans-retinoic acid (ATRA) induced HL-60 cell differentiation. Fibrates are pharmacological PPARalpha ligands commonly used in the treatment of hyperlipidaemia whereas MPA is used to treat breast carcinoma. We recently demonstrated that, potentiation of HL-60 differentiation by AKR1C3 inhibitors occurs via the consequent activation of PPARgamma. Thus combined CA and MPA (CA/MPA) provide the means to simultaneously target PPARalpha and gamma using established drugs with low systemic toxicities. Here we demonstrate that CA/MPA killed cultured primary AML blasts in the presence or absence of IL3 and SCF ($P = 0.0001$) but not normal CD34+ve myeloid blasts. CA/MPA also sensitized AML cell killing by suboptimal doses of daunorubicin and Ara-C ($P < 0.003$) highlighting the drugs' potential as chemosensitizing agents. Although ATRA did not enhance AML cell killing by CA/MPA, all three drugs together resulted in the differentiation of the surviving cells from three of seven non-acute promyelocytic AML samples. Finally, high serum Nm23-H1 protein concentrations have been associated with the poorest prognosis in AML. Here we provide the rationale for this by demonstrating that Nm23-H1 acts as an autocrine survival factor. Importantly we also demonstrate that CA/MPA rapidly down-regulated Nm23-H1 expression in those AMLs that had highest initial expression ($P = 0.001$). We conclude that CA/MPA exert multiple antileukemic actions against AML cells and should be considered as adjuncts in the treatment of elderly and relapsed AML.

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The utility of *in vitro* testing for imatinib sensitivity in BCR-ABL negative myeloproliferative disorders

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Imatinib mesylate (STI571, Glivec) inhibits the tyrosine kinase activity of the protein tyrosine kinases ABL, ARG, PDGFRA, PDGFRB and KIT, and has proven clinical efficacy in the management of patients with BCR-ABL positive CML and MPDs with PDGFRB rearrangements. However, it is not known to what extent patients with BCR-ABL negative MPDs may harbour activating rearrangements or point mutations of imatinib-responsive genes. To test for imatinib responsiveness we have employed two *in vitro* assays. First, blood or marrow mononuclear cells (MNC) were grown in methylcellulose supplemented with growth factors and treated with 0, 1 or 5 μM imatinib. Colonies were counted on days 7 and 14. Secondly, blood or marrow MNCs were placed in liquid culture with 0, 1 and 5 μM imatinib and cell numbers were enumerated over 2–3 weeks. Imatinib-treated cells from CML patients showed a reduction in colony and cell numbers over time compared with untreated cells and treated cells from normal individuals. We have obtained results using one or both methods in 38 BCR-ABL, TEL-PDGFRB negative patients who had been diagnosed with atypical CML, BCR-ABL negative MPD, CMML, mastocytosis, myelofibrosis or hypereosinophilic syndrome (HES). Eleven patients showed a positive response, four of whom had a PDGFRB rearrangement as determined by FISH and one had TEL-ABL. The remaining six patients had HES ($n = 2$), secondary MDS with eosinophilia following treatment for AML ($n = 1$), aCML ($n = 1$), MF ($n = 1$) or mastocytosis ($n = 1$). Six

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patients (five with PDGFRB or ABL rearranged MPD and one with HES) were treated and all showed a cytogenetic and/or haematological response. We conclude that *in vitro* cultures are useful for predicting patients who might respond clinically to imatinib. Evidence to date suggests that most BCR-ABL negative patients who are imatinib-responsive have elevated eosinophil counts.

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Identification of a novel gene, GEMS, that is fused to FGFR1 in the 8p11 myeloproliferative syndrome

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The 8p11 myeloproliferative syndrome (EMS) is an atypical stem-cell myeloproliferative disorder which is caused by activation of the transmembrane receptor tyrosine kinase FGFR1. This typically occurs as a result of fusion of the tyrosine kinase domain of FGFR1 to an unrelated partner gene, which promotes dimerisation and consequent activation of the kinase in the absence of an appropriate ligand. To date, five FGFR1 partner genes have been identified: ZNF198 at 13q12, FOP at 6q27, CEP110 at 9q33, BCR at 22q11 and HERV-K at 19q13. In this study, we describe a patient with a clinical picture typical of EMS, who was found to have an acquired ins(12;8)(p11;?p11p22) by cytogenetics. Southern blotting and FISH were used to confirm that FGFR1 was disrupted and RT-PCR analysis indicated that the patient did not harbour any of the known FGFR1 fusion mRNAs or BCR-ABL. To identify the partner gene in this patient we employed 5′-RACE PCR, exploiting the fact that all known FGFR1 translocations generate mRNA fusions in which the partner gene is spliced to FGFR1 exon 9. A 629-bp product was obtained which was found to consist of sequence derived from chromosome band 12p11 fused to exon 9 of FGFR1. Analysis of ESTs and other clones indicated that the sequence was derived from a widely expressed novel gene that we have called GEMS (gene disrupted in eight p11 myeloproliferative syndrome). GEMS is predicted to be translated into a 29-kDa protein that contains an N-terminal coiled-coil domain but no other recognisable motifs. RT-PCR confirmed the presence of the GEMS-FGFR1 fusion but the reciprocal chimaeric mRNA was not detected. Experiments to determine the transforming ability of this novel fusion gene are underway.

Oral Communication

Haemostasis and Thrombosis 2

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Venous thromboembolism (VTE) associated with the management of acute thrombotic thrombocytopenic purpura (TTP)

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VTE is reported to occur in 3% of TTP/HUS cases following plasma exchange (PEX) therapy. From May 1997 to May 2002, 68 patients with acute TTP were treated. Eight documented VTE episodes (six DVTs and three PEs) were identified in seven patients (all female, mean age 31 years) during therapy for acute TTP (five primary presentation and three relapses). All six DVTs were associated with central lines at the site of thrombosis. Other precipitating factors included pregnancy (one), immobility (eight), obesity (three) and FV Leiden heterozygosity (one). VTE occurred at a mean of 53 days following the first PEX. Solvent detergent plasma (Octaplas, Octapharma, Austria) was the last plasma to be used in PEX prior to the VTE in seven of eight events. Octaplas was used as the sole plasma for three TTP episodes and in the other episodes was substituted for cryosupernatant or FFP owing to poor response (four) or allergy (one). The mean volume of Octaplas replacement prior to the VTE was 100 L (range 22–206). The mean platelet count at VTE was 161 (range 12–232) $\times 10^9$/L. The platelet count had been in the normal range for a mean of 18 days prior to the VTE in those with a normal platelet count (six of eight). One patient received prophylactic LMW heparin from initial TTP diagnosis and seven of eight received aspirin. In conclusion, we identified VTE in 12% of TTP cases and 88% of these were associated with Octaplas replacement in PEX. VTE is a multifactorial disease and several known precipitating factors are present in TTP patients. The use of large volumes of Octaplas in PEX may be an additional risk factor, possibly associated with protein S (PS) deficiency, particularly as the median functional PS in Octaplas is 58 IU/dL compared with 91 IU/dL and 102 IU/dL in FFP and cryosupernatant respectively.

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Results of D-dimer in the UK NEQAS (Blood Coagulation) proficiency testing programme: urgent need for improvements in standardization of testing and reporting

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We report the proficiency testing of D-dimer in diagnostic departments. Two lyophilised samples were tested for D-dimer by over 240 centres using different methods. Around 30% used semi-quantitative tile-based latex agglutination assays and 70% used automated techniques including 20% who reported in fibrinogen equivalent units (FEU). Six automated methods were used in 10 or more centres and the median and range of results obtained with these methods are shown below in ng/mL. For sample 1, 70% of centres reported a result above the cut-off for DVT. For one method the median test result was within the normal range, whereas for other methods the median was 40–80% above the normal range depending on the technique. It was a major cause for concern that 24% reported a normal result for this sample. There appeared to be a poor understanding of the difference between the upper limit of normal and the cut-off for DVT diagnosis. For the 103 centres who reported using a cut-off for DVT diagnosis the value in use ranged from 80 to 1000 ng/mL. For sample 2, a pooled normal plasma, six centres reported their result as positive, abnormal or consistent with DVT. As reconstituted lyophilised plasma is unsuitable for the Nycocard system a different test sample was tested by 67 users of the Nycocard system who obtained results in the range of 80 to 1300 ng/mL for

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a liquid control sample having a median result of 500 ng/mL. We conclude that standardization of D-dimer testing and reporting is currently poor and that there is an urgent need for international reference preparations and recommendations.

		Sample 1		Sample 2		
	n	Median	Range	Median	Range	Median upper limit normal
Biomerieux VIDAS*	10	872	798–983	169	149–250	500
Dade-Behring DD Plus	10	170	120–219	100	60–170	190
Instrumentation Lab	68	352	240–848	103	10–280	260
Organon (FEU)*	18	915	770–4320	200	80–830	500
Organon (other units)	10	433	400–850	80	30–100	300
Stago*	15	890	800–1360	220	130–640	500

* FEU, fibrinogen equivalent units.

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Progression of liver disease in hepatitis C infected haemophiliacs assessed by serial liver biopsy

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More than 90% of haemophiliacs treated with plasma-derived factor concentrates prior to 1984 are infected with the hepatitis C virus. Although the timescale for progression to cirrhosis is many years, most patients will develop chronic liver disease. It has been suggested that liver biopsy in haemophiliacs is particularly hazardous and should be avoided. However, current treatment guidelines use liver biopsy to determine those who require therapy with ribavirin/interferon. We have previously reported our experience of liver biopsies in 35 patients with haemophilia/von Willebrand's disease and hepatitis C. We now report follow-up data on repeat biopsies on the same patient group 3–5 years following initial biopsy to assess progression of liver disease in this group. Patients who were HCV-PCR negative and those with inhibitors were excluded. To date, 25 patients have undergone repeat biopsy. Biopsies were graded according to fibrosis and inflammatory scores. Ten patients have progressed by one fibrosis grade. Three patients have progressed from grade 2 or less to grade 3 thereby qualifying for combination therapy. One patient has progressed from grade 0 to grade 3 and another progressed from grade 0 to grade 5. Both these patients had a history of excessive alcohol consumption. Thirteen patients showed no evidence of progression. Over a time course of at least 18 years only four patients have developed cirrhosis. There have been no bleeding complications related to the biopsies. We have performed 70 biopsies in patients with haemophilia/severe VWD and shown it to be a safe procedure. We have demonstrated the slow progression of liver disease in this group. The majority of our patients do not qualify for combination therapy according to national guidelines.

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UK surveillance of acquired haemophilia A: May 2001–April 2002

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Reports on acquired haemophilia (AH) are exclusively from referral centres which may have lead to publication bias. This study aims to establish the incidence and clinical characteristics of AH in all UK patients. In the first 12 months 256 of 257 UK Trusts circularised reported 98 cases (incidence 1.7/million/year). Clinical details are available on 68 patients. Median age is 80 (range 32–97). 39% had died at 6 months: 10% infection (pneumonia/sepsis), 4% bleeding, 7% malignancy, 7% cardiac and 11% others. Clinical associations are malignancy 29%, inflammation 20%, pregnancy 3%, pemphigoid 3% and none 59%. Median (range) factor VIII and anti-human inhibitor levels were 3 IU/dL (<1–25) and 13 BU/mL (<0.4–717). Bleeding manifestations are subcutaneous 78%, muscular 37%, gastrointestinal 22%, retroperitoneal 9%, haemarthrosis 7%, haematuria 7% and intracranial 3%. Subcutaneous bleeding was the only manifestation in 28%. Bleeding manifestations did not vary between CCC, haemophilia centres (HC) and other centres. Haemostatic treatment: none 38%, FEIBA 34%, rFVIIa 24%, human FVIII 22% and porcine FVIII 4%. No haemostatic treatment was required in 26% of patients at CCC, 55% at HC and 45% at other centres. Immunosuppression induced remission in 68% but only 44% of the cohort was alive and inhibitor-free at 6 months. There was no difference in remission rate between steroids alone 73% or cytotoxics and steroids 69% (IVIG made no significant difference); however, these results must be interpreted with caution as they are retrospective and not randomized. The remission rate at CCC, HC and other centres was 63, 65 and 91% respectively. These interim data suggest that overall survival is worse than previously reported, more patients died of infection than bleeding and many patients required no haemostatic treatment. Patients treated at non-haemophilia centres require less haemostatic treatment and are more likely to achieve remission suggesting that previously published data may be biased to a more severe phenotype.

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Procoagulant changes in human macrophages induced by environmental microparticles [PM10]: a possible pathogenetic mechanism in cardiovascular disease

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In epidemiological studies a strong relationship has been confirmed between increasing concentrations of environmental particles [PM10 and PM25] and acute cardiovascular events including myocardial infarction and stroke. No plausible pathogenetic mechanism

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has been demonstrated to explain this association. Recently it has been demonstrated that inhaled particles pass into the systemic circulation in humans (1). Therefore we hypothesized that vascular events may be triggered by coagulation activation on cell surfaces induced by contact with environmental particles. Human peripheral blood monocyte-derived macrophages and a human bronchial epithelial cell line were exposed to PM10 and supernatants examined for procoagulant activity in a plasma recalcification time assay. Control experiments employed culture medium only or hydrogen peroxide. Both PM10 and hydrogen peroxide induced an increase of procoagulant activity in culture supernatants from both cell types [shortening of clotting time versus control with PM10 was 31% with macrophages and 19% with epithelial cells; mean of three experiments, $P < 0.001$ and < 0.01 respectively]. In order to characterise the procoagulant activity from macrophages induced by PM10 we went on to determine the effects of these particles on surface expression of negatively charged phospholipid by annexin V binding, and tissue factor mRNA expression by RT-PCR. Finally we measured macrophage interleukin 8 (IL8) mRNA expression and release in response to PM10. Environmental particles induced macrophage tissue factor mRNA expression and significantly increased bound annexin V. Furthermore IL8 message and release were significantly enhanced also. We have demonstrated, for the first time, that environmental particles associated with increased cardiovascular risk can activate macrophages *in vitro* with transformation to a procoagulant phenotype which may be mediated through apoptosis/negatively charged phospholipid exposure and tissue factor expression. This may represent a novel prothrombotic mechanism triggered when environmental particles reach the systemic circulation.

Reference

1 Nemmar *et al. Circulation* 2002; **105**: 411–4.

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Is early activation of the clotting cascade involved in the pathogenesis of verotoxin-producing *Escerichia coli*-associated haemolytic uraemic syndrome?

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In most cases, haemolytic uraemic syndrome (HUS) is a thrombotic complication of infection with *Escherichia coli* O157 : H7. It usually follows bloody diarrhoea caused by verotoxin secreted by the bacteria, and comprises acute renal failure, non-immune, microangiopathic haemolytic anaemia and thrombocytopaenia. HUS is primarily a disorder of the vascular endothelium, with platelet aggregation at sites of endothelial cell damage in the renal microvasculature. Several *in vitro* studies demonstrate changes in endothelial cell anticoagulant properties when exposed to cytokines and under apoptotic conditions. Of interest are changes in tissue factor (TF) expression and activity, TF being crucial in initiating the coagulation cascade. Additionally, the expression of antithrombotic membrane components, notably thrombomodulin, has been shown to be downgraded. We examined the changes in TF and thrombomodulin on endothelial cells exposed to the *E. coli* products, verotoxin and lipopolysaccharide (LPS), and the inflammatory cytokines, interferon-gamma (IFNgamma) and tumour necrosis factor-alpha (TNFalpha), and their relationship to apoptosis. Macrovascular [primary culture of human umbilical vein endothelial cells (HUVEC)] and microvascular [human microvascular endothelial cells (HMEC)] endothelial cells were cultured with LPS and verotoxin in the presence and absence of cytokines. Surface TF and thrombomodulin expression and apoptosis were assessed using flow cytometry. Increases in TF expression were observed in activated HUVECs; changes were less apparent in HMECs which constitutively express TF. Thrombomodulin was consistently reduced on HMECs under similar conditions. It appeared not to be the apoptotic population that changed their expression of membrane proteins. HUVECs cultured with LPS and TNFalpha demonstrated the greatest increase in TF. Decreases in thrombomodulin were greatest on HMECs exposed to verotoxin. We demonstrate an up-regulation of TF and a down-regulation of thrombomodulin on endothelial cells stimulated with LPS, verotoxin and inflammatory cytokines. These cells are rendered more thrombogenic, mechanisms that may be involved in the pathogenesis of HUS.

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Paediatric and General Haematology

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Revealed: D-dimers are being inappropriately requested. Results of audit to influence subsequent requesting

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D-dimers, cross-linked fibrin degradation products, are extremely sensitive, but non-specific markers for coagulation activation; a process that complicates many conditions. They have been shown to be useful in negatively predicting for deep vein thrombosis (DVT), particularly in combination with a clinical assessment score for DVT. Junior medical staff and GPs are increasingly using the D-dimer test to screen for the presence of DVT. Radiology departments use a positive D-dimer to 'gate-keep' their Doppler service. Haematology departments have therefore identified a dramatic increase in D-dimer, requesting and associated cost (£26,000 in 2002 at Derriford Hospital). Over-investigation may lead to unnecessary, and potentially dangerous, further investigation and anticoagulation. This audit was conducted to address these concerns. The case-notes were reviewed from 50 consecutive patients presenting to/within Derriford Hospital with suspected DVT (34%) or pulmonary embolism (66%) in 2002. Not surprisingly, junior-most of the doctors requested the bulk of these investigations, 11% by registrars and none by the consultants. The reasons for requesting were documented in all but seven patients. The Biomerieux MDA D-dimer assay kit was previously evaluated locally, giving a positive cut-off level of 0.5 mg/mL for DVT with >95% sensitivity. In the current group of patients, 28% fell below the cut-off value. Of the positive results, only 61% were investigated further. The management was not affected by the D-dimer result in 45% of cases. Of the original cohort, only four DVTs and two PEs were confirmed. One PE was diagnosed in spite of negative D-dimer. These data support the theory regarding over-requesting of D-dimers. The low specificity is highlighted by only six confirmed DVT/PEs of 72% patients having a raised D-dimer. Locally, the co-importance of validated clinical assessment score is emphasized and the futility/danger of performing D-dimers on

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in-patients and those with suspected PE until a specific cut-off value has been established.

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Anti-D immunoglobulin in the treatment of immune thrombocytopenic purpura in adults in the United Kingdom: an evaluation of clinical outcomes and resource utilization

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Immune thrombocytopenic purpura [ITP] is an autoimmune disorder distinguished by a low platelet count and mucocutaneous bleeding. Estimated incidence is 10 per 100,000 persons per year. ITP is classified as primary, or secondary to an underlying disorder, and acute (of 6 months or less in duration), or chronic. In 70% of children, resolution occurs in 6 months. By contrast, ITP in adults has an insidious onset, and is chronic in nature. Given comparable efficacy of anti-D immunoglobulin and IVIg in the treatment of ITP, this inquiry was designed to contrast health resource utilization patterns stemming from the use of each agent in the treatment of ITP in adults. Stemming from the schematic developed by Cines and Blanchette (1), and information contained in the report of the British Committee for Standards in Haematology, General Haematology Task Force (Guidelines for the investigation and management of idiopathic thrombocytopenic purpura in adults, children, and in pregnancy) we employed Monte Carlo simulation to evaluate the utility (cost implications) of various treatment schema (intent-to-treat format; steroids; IVIg; anti-D immunoglobulin) for the treatment of ITP. Utilizing 100,000 replicate simulations per year of age examined [15 through 80 years (5-year intervals); quadratic regression equations] we discerned a significant ($P < 0.05$) efficacy/health service utilization-based saving (percent reduction) for the 'steroids to anti-D immunoglobulin' arm, relative to the 'steroids to IVIg' arm [34.0% (SE = 0.1%) for males; 33.4% (SE = 0.1%) for females]. In the United Kingdom, use of anti-D immunoglobulin in the management of ITP is estimated to result in reduced health service expenditures relative to IVIg. Specifically, use of anti-D immunoglobulin reduces the time required (cost) for medication administration, patient monitoring, and occupancy of health care facilities. The effect on patient's quality-of-life warrants further research.

Reference

1 Cines and Blanchett. *NEJM* 2002; 346: 995–1008.

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Reduced numbers and antigen unresponsiveness of natural killer T-cells in idiopathic (autoimmune) thrombocytopenia (ITP)

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Natural killer T cells (NKT cells) are a rare cell subset implicated in the control of autoimmunity. Here, untreated ITP patients and healthy controls were compared for the number of circulating NKT cells and their proliferative response to the NKT cell antigen alpha-galactosylceramide. The cells were identified as Valpha24 Vbeta11 double-positive (CD4 positive or CD4 CD8 double-negative) lymphocytes using flow cytometry. In controls, NKT cell frequency ranged from 0.008 to 0.351% of total lymphocytes (mean 0.13%, $n = 9$). In the ITP group, a low NKT cell frequency was much more common, resulting in a mean of 0.04% (range 0.002–0.336%, $n = 14$). The capacity for alpha-galactosylceramide-driven NKT cell expansion was variable in the ITP group, which could be divided into responders (55% of tested donors, $n = 13$) and non-responders (45%). In comparison, 92% of controls ($n = 13$) responded. Interestingly, repeated testing of a limited number of ITP patients showed that proliferative response to the antigen or unresponsiveness was not constant over time, thus one and the same patient may respond at one time but not another. Furthermore, while IL-2 increased antigen-induced NKT cell proliferation in the majority of controls, this was not observed in the ITP group. In conclusion, the number of circulating NKT cells is reduced in ITP and a proportion of ITP patients have both antigen and IL-2 unresponsive NKT cells. The lack of proliferative response after antigen exposure and the IL-2 unresponsiveness may be due to adaptive tolerance, or *in vivo* anergy. This is believed to be caused by a prior incomplete antigen activation *in vivo*. These observations therefore suggest that NKT cells from ITP patients are anergic and this may contribute to the autoimmune process. Future research is planned to assess NKT cell function and involvement in autoimmune thrombocytopenia.

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Outcome heterogeneity in childhood high hyperdiploid acute lymphoblastic leukaemia

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High hyperdiploid (HeH) karyotypes (51–65 chromosomes) are found in approximately one-third of children with acute lymphoblastic leukaemia (ALL). Generally, these patients respond well to standard therapy and enjoy a superior outcome compared with other children. It has been suggested that specific cytogenetic features might further refine this prognosis and provide a method for identifying those with a poorer outlook, however no consensual picture has yet emerged. We analysed outcome data from 700 HeH ALL children enrolled on two MRC trials (UKALLXI and ALL97) to examine the effect of sex, age, white blood cell (WBC) count, individual trisomies, modal number and structural abnormalities on survival. Univariate analysis showed that age (1–9 years), sex (female), trisomies of chromosomes 4, 10 and 18, and a modal number of 54–65 chromosomes were all associated with improved survival, whereas the presence or absence of structural abnormalities and WBC had no effect on outcome. Multivariate analysis revealed that only age, sex, trisomy 4 (+4) and trisomy 18 (+18) were independent indicators of outcome. These results enabled the construction of two hazard scores for predicting relapse and mortality. Three risk groups were found using age, sex and +18, with event-free survival (EFS) rates of 86, 75 and 50% at 5 years ($P < 0.0001$). The group with the highest risk of relapse comprised:

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boys aged >9 years, boys aged 1–9 years without +18 and girls >9 years without +18. While girls aged 1–9 years with +18 made up the group with the best EFS. In terms of mortality, those aged <10 years with both +4 and +18 had a significantly improved survival (96 versus 84% at 5 years, $P < 0.0001$). These findings confirm that the outcome of children with HeH ALL is heterogeneous and information regarding specific trisomies can help identify those with a greater risk of treatment failure.

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Correction of the anaemia of chronic disease (ACD) with small doses of recombinant erythropoietin

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The anaemia associated with chronic disease such as infectious, inflammatory or neoplastic conditions, contributes significantly to morbidity. It has been recognized recently that a similar anaemia is also seen in congestive cardiac failure (CCF) and diabetes without significant renal failure. Pharmacological doses of recombinant erythropoietin (EPO) correct ACD associated with rheumatoid arthritis, inflammatory bowel disease, and AIDS. Good responses to EPO have also been seen in cardiac failure, and type 1 diabetes. Large doses of EPO are used in some centres to treat anaemia associated with cancer and its treatment. We corrected ACD with small doses of EPO in 10 patients: six males and four females, age range 58–88 years. Three patients had inflammatory disease, three were diabetic, and four had CCF. Six had either normal or reactive bone marrow histology, normal cytogenetics, and normal or increased iron stores. Six were transfusion dependent prior to EPO therapy. The dose of subcutaneous recombinant human erythropoietin (Eprex) was titrated according to the haemoglobin. Nine patients were evaluated, one having died from his underlying disease. The mean dose required was 9000 units/week (range 3000–14,000). The average maximum increase in haemoglobin was 4.1 g/dL (range 1.3–6.8). Few side effects were encountered. One patient suffered transient cerebro-vascular ischaemia, possibly related to a rapidly rising haemoglobin. All patients became transfusion independent. All reported improved general health without the peak and trough effect associated with blood transfusion. Avoidance of red cell transfusion is desirable in view of the possible infective and non-infective complications of transfusion and concern over the availability and cost of blood products. Although EPO is expensive, the doses required were comparable with those used in chronic renal failure, and much smaller than usually required to treat anaemia associated with cancer and its treatment. The cost therefore, compared favourably with blood transfusion.

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Epidemiological evidence for an infectious origin for childhood leukaemia

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The aim of the study was to interpret the results of several analyses of childhood leukaemia data from the Manchester Children's Tumour Registry (MCTR), in view of current hypotheses regarding an infectious origin. The MCTR is population-based and has been collecting case data from a geographically defined region of North West England since 1954. Trend tests, space-time clustering tests (Knox and K-function), ecological regression and geographical mapping were used to study the data on the leukaemias, broken down by sub-type (ALL, AML). For the most recent period (since 1980), ALL was further examined by immunophenotypic sub-type. There was an increasing incidence of ALL, due mainly to an increase of the precursor B-cell sub-type in the childhood peak. Space-time clustering was evident for cases of ALL, aged 0–4 years, for the time period 1954–1985, mainly based on date of diagnosis and place of birth. For the time period 1980–2001, space-time clustering was confined to cases of precursor B-cell ALL, aged 18–54 months, based on date and place of birth. The incidence of ALL was greater in more densely populated wards, largely because of cases of non-precursor B-cell ALL. The studies present a complex picture, but are supportive of an infectious aetiology, particularly for precursor B-cell ALL. It is concluded that there may be at least two infectious mechanisms operating, with a different mechanism predominating in the earlier years from that in the later years.

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The NEW ENGLAND JOURNAL of MEDICINE

ORIGINAL ARTICLE

Effect of Eculizumab on Hemolysis and Transfusion Requirements in Patients with Paroxysmal Nocturnal Hemoglobinuria

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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.

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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.

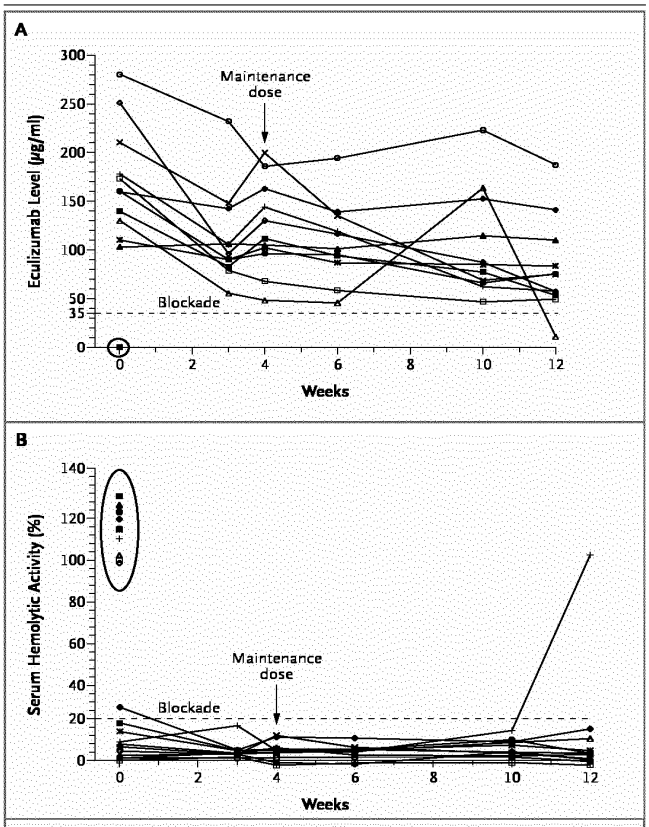


Figure 1. Pharmacokinetic and Pharmacodynamic Analysis of Eculizumab in 11 Patients with Paroxysmal Nocturnal Hemoglobinuria (PNH).

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).

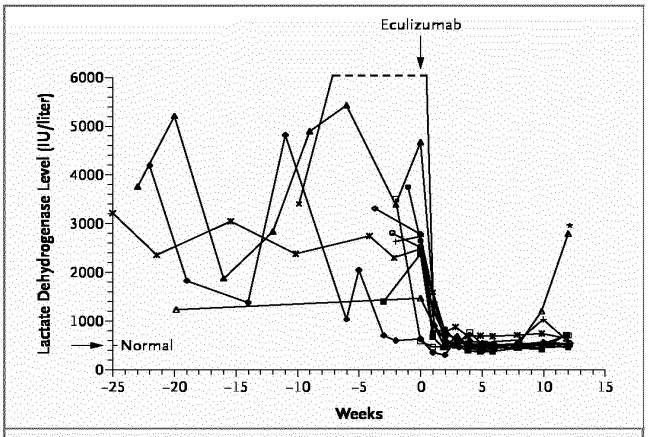


Figure 2. Analysis of Lactate Dehydrogenase Levels, a Biochemical Indicator of Hemolysis, in 11 Patients with Paroxysmal Nocturnal Hemoglobinuria up to 25 Weeks before and during 12 Weeks of Eculizumab Treatment.

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).

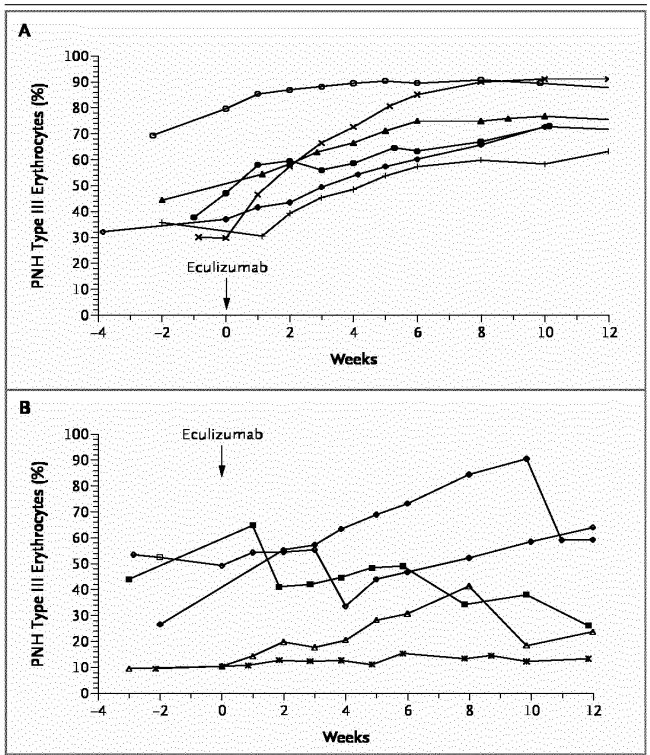


Figure 3. Changes in the Percentage of Paroxysmal Nocturnal Hemoglobinuria (PNH) Type III Erythrocytes during Eculizumab Treatment in Six Patients Who Received No Transfusions during Treatment (Panel A) and Five Patients Who Received at Least One Transfusion after Starting Treatment (Panel B).
The initial 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

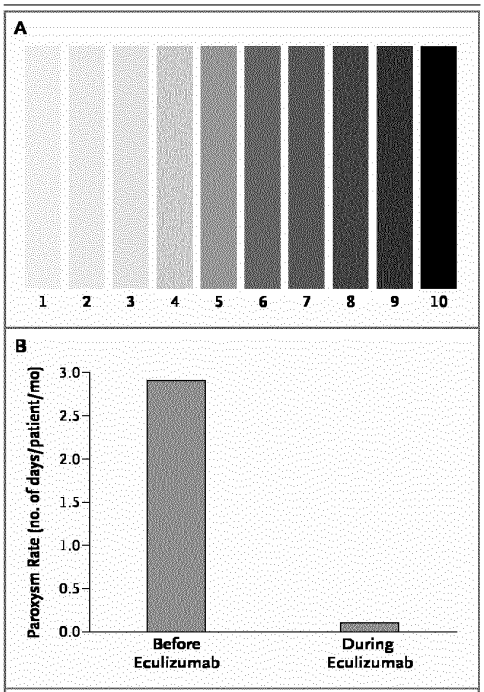


Figure 4. **Incidence of Paroxysms during Eculizumab Treatment.**

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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D4

Eculizumab

Prop Inn; USAN

Humanized Anti-C5 Monoclonal Antibody

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

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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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BRIEF REPORT

Recurrence of Neisserial Meningococcemia Due to Deficiency of Terminal Complement Component

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INHERITED AND ACQUIRED DEFECTS of the complement system predispose susceptible individuals to infections with *Neisseria meningitidis*.[1,2] Deficiencies of the terminal complement components (C5, C6, C7, or C8) are mainly associated with mild recurrent meningococcal infections in patients usually more than 10 years old at the time of the first infection.[1,2] The prevalence of complement deficiencies in patients with meningococcal disease ranges from 0 to 30%,[1,3,4] whereas in a white general population the prevalence is estimated to be less than 0.03%.[1,2] *Neisseria meningitidis* infections due to the common serogroups (A, B, C) are seldom associated with complement deficiencies.[1,2,4] Rasmussen et al[5] found no complement deficiencies in any of 132 patients with serogroup B infections. We report the case of a patient with recurrent neisserial meningococcemia due to deficiency of the sixth component of complement.

CASE REPORT

A 21-year-old black man who had had no pertinent past illnesses, came to the emergency room complaining of a severe headache. During the evaluation his mental status deteriorated to the point that he responded only to noxious stimuli. He was subsequently given 4 million units of penicillin G for meningitis and was admitted. His admission temperature was 38.3°C (101°F) rectally, pulse rate 50/min, blood pressure 160/98 mm Hg, and respiratory rate 14/min. Both pupils measured 5 mm and responded sluggishly to light. Nuchal rigidity and Kernig's and Brudzinki's signs were present. Results of lung, cardiac, and abdominal examinations were normal. Petechiae measuring 1 to 2 mm covered the chest, legs, and soles of the feet. Serum chemistry values were sodium 136, potassium 4, chloride 101, and bicarbonate 20 mEq/L; glucose 152, BUN 14, and creatinine 1.4 mg/dL. WBC was 21 600/mm^3 with 67% neutrophils and 22% band forms, and the hematocrit value was 45%. Lumbar puncture showed an opening pressure of 50 cm, and Gram's stain of the purulent CSF showed multiple gram-negative diplococci consistent with *Neisseria meningitidis*. The CSF protein value was 660 and glucose value, 0 mg/dL. Penicillin G therapy was continued at 2 million units every 2 hours intravenously, and 2 doses of methylprednisolone were given (2 g every 6 hours). The patient's mental status returned to normal over the following 72 hours. He received a 14-day course of penicillin G, and rifampin was given to his roommates, household contacts, and girlfriend. The *N meningitidis* was stereotyped as group B. There was no evidence of adrenal insufficiency at discharge from the hospital.

The patient did well until 3 weeks after discharge when he was admitted for evaluation of diffuse myalgias, right-sided neck stiffness, sore throat, and a temperature of 39°C (102.3°F). Physical examination showed no nuchal rigidity or Kernig's sign. WBC was 19 300/mm^3 with a left shift. Lumbar puncture yielded CSF containing 3 WBCs and 13 RBCs/mm^3, with a glucose value of 95 and a protein value of 17 mg/dL. Over the next 24 hours increasing myalgias developed, in addition to palatal petechiae and purpura on the palms and soles. He was given penicillin, gentamicin, and methylprednisolone for possible Waterhouse-Friderichsen syndrome. Blood cultures grew *N meningitidis* group B. Complement determinations showed normal C3, C4, C5, C7, and C8 levels but a CH_{50} of 0 CH U/mL and a greatly reduced C6 level of <1.5 mg/dL (normal 4.8 to 7.2 mg/dL). The patient received a 14-day course of penicillin G for treatment of recurrent neisserial bacteremia. He also received pneumococcal and *Haemophilus influenzae* vaccinations and 2 days of rifampin therapy for elimination of a possible *N meningitidis* carrier state. Methylprednisolone therapy was discontinued after 48 hours when no signs of Waterhouse-Friderichsen syndrome appeared.

DISCUSSION

The alternate and classical pathways of the complement system are important components of the host's immune response to infections caused by encapsulated organisms. Deficiencies of complement components have been associated with recurrent infections due to specific organisms. A number of studies have demonstrated the association of complement deficiencies and *Neisseria meningitidis* infections.[4,6-12] Our patient has C6 deficiency as a cause of his recurrent bacteremia with *Neisseria meningitidis* serogroup B. He had had no previous episodes of neisserial infections.

The median age of first infection in patients with late manifestation of complement deficiency has been reported to be 17 years, despite the presence of the defect since birth and the early peak age-specific incidence of meningococcal disease in the general population (ages 3 to 8 months).[2] Recurrent infections are not unusual with discrete recurrences reported in 45% of complement deficient individuals.[2]

Our patient had recurrent infections with *Neisseria meningitidis* serogroup B. This is an infrequent situation, since recurrent infections with *Neisseria meningitidis* are more commonly caused by serogroup Y than by the common serogroup B.

SUMMARY

Recurrent infections with *Neisseria meningitidis* are attributed to deficiencies of terminal complement components. The sero-

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type most commonly responsible for recurrent *N meningitidis* infections is serotype Y. We have reported a case of recurrent meningitis due to *N meningitidis* in a patient who was found to be deficient in the sixth component of complement. Complement deficiencies should be considered in any patient with recurrent infections caused by *N meningitidis*.

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Case report

Complement C7 deficiency presenting as recurrent aseptic meningitis

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Background: Complement deficiency states are rare inherited disorders that may predispose affected individuals to angioedema, collagen vascular disease, or infection due to encapsulated organisms, especially *Neisseria meningitidis*.

Objectives: To report the case of a 36-year-old man of Irish descent with recurrent culture-negative neutrophilic meningitis, to offer potential reasons for the inability to recover a causative pathogen, and to review the genetics and prevalence of complement deficiency states, the methods of screening for such deficiencies, the features of meningococcal infection as they relate to such deficiencies, and management strategies for clinicians caring for patients with such deficiencies.

Methods: The patient presented in 1988 and again in 2002 with culture-negative neutrophilic meningitis. His second episode was characterized by a rash suggestive of meningococcal infection, prompting immunologic evaluation.

Results: Immunologic evaluation revealed an undetectable CH_{50} level. Levels of C1, C2, and C5 through C9 were normal except for C7, which was undetectable. Further testing revealed that the patient's sister was also C7 deficient.

Conclusions: Complement component deficiencies are relatively rare; individuals with collagen vascular disease and systemic neisserial infection should be screened using either the CH_{50} or the APH-50 assay. Key to the management of a late–complement component–deficient host is counseling, education about meningococcal infection, and discussions about the potential benefits of chemoprophylaxis and immunoprophylaxis. The ability to detect the bacterial cause of meningitis in such patients is organism dependent and may be influenced by factors such as cerebrospinal fluid bacterial concentration and previous antibiotic drug exposure.

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INTRODUCTION

The complement pathways play an integral role in the immune system.[1,2] Deficiencies in any complement components or pathologic activation of a normally functioning complement system can yield clinical disease, typically manifested as angioedema, collagen vascular disease, or infection. The seventh component of complement, C7, is part of the membrane attack complex (MAC) required for complement-dependent bactericidal activity, including defense against neisserial infection.[3,4] In this article, we describe a patient with C7 deficiency presenting with inflammatory arthritis and recurrent culture-negative neutrophilic meningitis.

CASE REPORT

A 36-year-old man was well until April 2002, when he developed transient pharyngitis followed by fever (temperature, 102.2° F), chills, sweats, and malaise. Subsequently, he developed headache, light sensitivity, neck pain and stiffness, nausea, vomiting, and right hip pain with an overlying rash. Five days into his illness, he presented to the emergency department (ED) with ongoing symptoms and a rash now involving his left shoulder, chest, and abdomen.

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The patient was hospitalized 14 years earlier for an illness consistent with bacterial meningitis by clinical presentation and lumbar puncture (LP) but with negative cerebrospinal fluid (CSF) and blood culture findings. He takes no medications, including nonsteroidal anti-inflammatory agents, and has no history of craniofacial trauma or drug allergies. His father died at the age of 45 years of a myocardial infarction. There is no family history of immunodeficiency, meningitis, arthritis, or other inflammatory or inherited disease. He lives with his wife and daughter in Connecticut, smokes cigarettes, and drinks alcohol, but he has never used illicit drugs. He is monogamous with his wife and has no history of sexually transmitted diseases. He is a plumber, has not traveled outside New England, and has no history of tick exposure. He did have contact with rodent droppings 1 day before becoming febrile.

In the ED, the patient appeared ill. His temperature was 101.0° F and his heart rate was 110/min. His blood pressure was normal without orthostatic changes, and his respiratory rate and oxygen saturation were normal. Nuchal rigidity and photophobia were present. Neurologic examination revealed only a positive Brudzinski sign. He had dry mucous membranes, flat neck veins, and no lymphadenopathy. His lungs were clear, his heart was tachycardic, and the results of abdominal examination were normal. There was markedly limited range of motion of the right hip and left shoulder. The

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affected joints were warm but without appreciable erythema or effusion. A scattered, tender, purpuric-petechial rash was distributed over the chest, left shoulder, arms, abdomen, right hip, and groin. The palms and soles were spared.

The leukocyte count was 18,000/mL, with 62% segmented forms and 30% bands; the hematocrit level was 43.4%; andthe platelet count was 228,000/mL. Findings from a comprehensive metabolic profile, coagulation indices, and urinalysis were unremarkable. Findings from chest radiography were normal, and electrocardiography showed sinus tachycardia. Lumbar puncture disclosed hazy CSF, with an opening pressure of 123 mm H_2O, a protein level of 186 mg/dL (normal, 14–45 mg/dL), a glucose level of 49 mg/dL (normal, 45–75 mg/dL), a red blood cell (RBC) count of 128/mL, a white blood cell (WBC) count of 491/mL (90% neutrophils), and moderate polymorphonuclear cells but no organisms on Gram stain.

One hour after ED arrival, a blood sample was cultured, and then 1 g of ceftriaxone was administered. One hour later, LP was performed, and then 1 g of vancomycin and an additional 1 g of ceftriaxone were administered, and a second blood sample was cultured. The patient was placed on "droplet precautions" in the intensive care unit, vigorously hydrated, and empirically treated for bacterial meningitis with ceftriaxone and vancomycin. His meningitic symptoms resolved and his vital signs normalized within 12 hours, whereas his arthritic symptoms recovered more slowly. Left shoulder aspirate yielded 4 mL of frank pus, a WBC count of 234,133/mL (90% neutrophils), and an RBC count of 800/mL. Right hip aspirate was serosanguinous, with a WBC count of 25/mL (45% neutrophils) and an RBC count of 24,747/mL. No crystals or organisms were identified from either specimen. Skin lesion Gram staining was negative for organisms. One of 2 bottles from the first blood culture grew coagulase-negative staphylococcus. Both bottles from the second blood culture were negative. Cerebrospinal fluid, skin lesion, and synovial fluid cultures were negative. Serologic test results for Lyme disease, Rocky Mountain spotted fever, and leptospirosis were negative. Findings from CSF latex agglutination for *Streptococcus pneumoniae, Neisseria meningitidis,* and *Haemophilus influenzae* were negative. The CH_{50} level, determined by enzyme colorimetry, was undetectable at less than 10 U/mL (normal, 26–58 U/mL; Quest Diagnostics Reference Laboratory, Wallingford, CT). Levels of C1, C2, and C5 through C9 (protein quantitation by radial immunodiffusion) were normal except for C7, which was undetectable at less than 2 mg/dL (normal, 4–11 mg/dL; Quest Diagnostics Reference Laboratory). C1 esterase inhibitor, antinuclear antibody, antibody to double-stranded DNA, rheumatoid factor, quantitative immunoglobulin, and human immunodeficiency virus antibody levels were all normal.

The patient completed 14 days of parenteral antibiotic drug therapy and was given the tetravalent meningococcal vaccine and a medical identification bracelet (MedicAlert; MedicAlert Foundation International, Turlock, CA) before hospital discharge. CH_{50} and C7 levels measured during his convalescence remained undetectable. Family members were screened for complement deficiencies (Table 1).

DISCUSSION

Genetically determined complement deficiencies have been described for nearly all of the complement components, including C7, which is inherited in an autosomal-recessive manner.[3] The C6 and C7 genes are polymorphic, closely linked on 5p13, and are close homologues of the C8α, C8β, and C9 genes.[5] The molecular bases of subtotal and total C6 and C7 deficiency have been reported previously.[6,7] Numerous polymorphic DNA markers have been reported for the C6 and C7 genetic region, illustrating the genetic heterogeneity associated with defects in these complement components.[8–15] Such polymorphisms have been described in individuals of Japanese, South African, Russian, Moroccan Sephardic Jewish, and Irish ancestry. The patient's parents emigrated from Ireland, and both must have been carriers for C7 deficiency. His father is deceased, but his mother's serum C7 level was compatible with a carrier state. Further testing revealed that his sister is also C7 deficient (Table 1).

Complement deficiency states are typically identified via the CH_{50} or the APH-50 assay. All of the terminal components of complement (C5-C9) are required to yield normal CH_{50} and APH-50 levels; thus, both assays provide a sensitive means of screening for late–complement component–deficient (LCCD) hosts.[4] Using these assays, the prevalence of complement deficiency states in the general population has been estimated to be 0.03%.[16] Given the rarity of complement

Table 1. Results of Complement Studies Performed on the Proband and His Family Members

Source	Age, y	Health status	Complement, mg/dL								
			CH_{50}*	C2	C3	C4	C5	C6	C7	C8	C9
Proband	36	Normal	<10	3.4	211	25	22	10.6	<2	8.6	73
Daughter	5	Normal	53	2.1	113	17	10	6	6	5.5	24
Brother	39	Diabetes	60	3	117	16	14	6.6	4	5.6	29
Sister	38	Normal	<10						<2	8.7	44
Mother	61	Normal	48	2.5			15	6	2	4.6	29
Paternal aunt	62	Normal	51	2.8			17	3.6	4	5	25
Normal range			26–58	1.6–3.5	88–201	16–47	6–20	3.6–7.5	4–11	2.7–6	6–29

* Measured in units per milliliter.

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deficiency states, the utility of screening assays is greatest in disease populations known to contain the clinical correlates of complement protein abnormalities (patients with collagen vascular disorders and those with systemic neisserial infection).[17] For patients with meningococcal disease, the prevalence of an underlying complement deficiency state varies worldwide (10%–15% in the United States) and is inversely related to the incidence of meningococcal disease by country.[3,17] Such relationships must be considered when making recommendations regarding the value of screening an individual patient with meningococcal infection for an underlying complement deficiency. A CH_{50} level was obtained on our patient because of his history of recurrent meningitis and a rash that was most consistent with meningococcal disease. Once the CH_{50} level indicated a complement deficiency, assays for the individual complement components were performed, and a C7 deficiency was identified. The patient's family was also screened using the CH_{50} assay and then the targeted C7 assay (Table 1).

Neisseria meningitidis causes approximately 80% of the infections in patients with any complement deficiency, and the remainder are due to other encapsulated organisms, such as *S pneumoniae* and *H influenzae*.[17] Infections in LCCD hosts, though, are nearly exclusively due to *N meningitidis*. This can be explained by understanding that the terminal complement components compose the MAC, which is responsible for complement-mediated bacterial cell lysis. Patients who are LCCD, therefore, are unable to assemble the MAC and are unable to effect complement-mediated cell lysis. *Neisseria meningitidis* (in contrast to all gram-positive and some gram-negative organisms) is extremely sensitive to complement-mediated killing,[3] and, thus, LCCD patients are particularly susceptible to meningococcal infection.

The frequency of meningococcal disease is 10,000-fold greater in LCCD hosts vs healthy hosts (57% vs 0.0072%).[17] Furthermore, in LCCD hosts there is a later median age of first meningococcal infection (17 vs 3 years), a higher relapse rate (7.6% vs 0.6%), a higher recurrence rate (41% vs 0.34%), and a 10-fold lower mortality rate (1.5% vs 19%) compared with healthy hosts.[17] Ross and Densen[3] explain these differences using hypothetical relationships among serum bactericidal activity, protective antibody, meningococcal exposure, and age. According to this model, both LCCD and healthy newborns express high levels of maternal protective antibody and serum bactericidal activity. Thereafter, both of these levels fall to their nadir such that, in healthy hosts, there is a narrow window of susceptibility for meningococcal infection between 6 months and 2 years of age. During this window, asymptomatic exposure to *N meningitidis* begins to occur, yielding a rise in serum bactericidal activity and protective antibody. In the LCCD host, serum bactericidal activity remains undetectable, yielding a lifelong, broad window of susceptibility to meningococcal infection. This lifelong window of susceptibility explains why LCCD patients are at increased risk of meningococcal infection, have an older median age of first infection, and have higher recurrence rates compared with healthy hosts.

In terms of the lower mortality rate noted in LCCD patients, some researchers[18] attribute this to ascertainment bias (complement deficiency is usually diagnosed in survivors of meningococcal disease, and complement testing is rarely performed in patients with fatal disease). Those who believe that this difference in prognosis is real offer 2 possible explanations. First, the lack of a MAC, as seen in LCCD hosts, results in preservation of the bacterial cell membrane, and, thus, there is no endotoxin release, septic shock, or death.[18] Second, in contrast to the sporadic cases in healthy hosts, episodes of meningococcal infection in LCCD patients occur in the presence of protective antibody, thus allowing opsonization to occur, which may clear some of the organism by phagocytosis.[3] Assuming that our patient had meningococcal infection at the age of 22 years, and again now, his older age at the first episode, recurrent disease, and survival are all typical features of meningococcal infection in an LCCD host.

Presumed and confirmed meningococcal infection in C7-deficient hosts has been previously reported,[3–8,18–33] mostly in the form of case reports containing incomplete clinical details. Review of 24 C7-deficient patients with a first episode of presumed or documented meningococcal infection reveals no sex preference and a median age of 15 years (range, 3–40 years).[5,7,8,18,22–33] Patients' ethnic origins include Irish, Moroccan, Russian, Japanese, Arab, and Yugoslav. Twenty (83%) of the 24 patients had clinical or laboratory evidence of meningitis either alone or in conjunction with arthritis (n = 4; 17%) or myopericarditis (n = 2; 8%). Three patients had chronic meningococcemia without central nervous system involvement. Rash was reported in 12 patients, and it was characterized as petechial or purpuric in 11 of the 12 patients. The CSF formula was not provided for these 24 patients, but up to 20% of cases of meningococcal meningitis from the literature have a CSF WBC count less than 1,000/mL, and 24% to 55% have a normal CSF glucose concentration.[34–37] Of the 24 C7-deficient patients we reviewed, the clinical diagnosis of meningococcal infection was confirmed by bacterial culture in 15 (63%). Even lower rates of microbiologic confirmation (20%–40%) were reported for 77 episodes of meningococcal infection in 30 patients with either C7 or C8 deficiency[18]; the authors attribute this to exposure to high-dose penicillin 1 to 2 hours before blood and CSF cultures were obtained. Finally, 12 of the 24 C7-deficient patients we reviewed had recurrent meningococcal infection, 3 had multiple recurrences, and all 24 responded to parenteral antibiotic drug therapy without clinical sequelae. Therefore, our patient's age, ethnicity, clinical presentation (now and 14 years ago), CSF formula, and response to therapy are all compatible with recurrent meningococcal infection in a C7-deficient host.

Although it is suggestive that our patient had meningococcal infection, no such organism was identified using routine methods. The possibility remains that he was infected by a bacterium other than *N meningitidis* or by a nonbacterial pathogen or that he experienced a noninfectious meningitis syndrome. Tick-borne bacterial infections, such as Lyme disease, Rocky Mountain spotted fever, and ehrlichiosis, typ-

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ically occur in the late spring and summer months and can manifest as aseptic lymphocytic meningitis with or without joint involvement. Our patient exhibited several features that did not support the diagnosis of a tick-borne infection: his illness occurred in April, he had no history of a tick bite, his rash differed from the characteristic rash of Lyme disease and Rocky Mountain spotted fever, his arthritis developed during the acute phase of illness (as opposed to Lyme arthritis, which is a late manifestation), and he had neutrophilic CSF pleocytosis. Results of short-term serologic testing for the agent of Lyme disease and Rocky Mountain spotted fever were negative. The CSF and Lyme antibody and Lyme polymerase chain reaction on the synovial fluid, however, was not performed. Aseptic meningitis can also be a manifestation of leptospirosis and lymphocytic choriomeningitis. Such infections typically occur during the second phase of disease, 1 to 2 weeks after exposure to rodents or rodent droppings. Our patient's exposure to rodent droppings occurred only 1 day before he became ill, thus making these infections very unlikely. Noninfectious processes that have been associated with aseptic meningitis include collagen vascular disorders, medications,[38] adult-onset Still disease,[39] and complement deficiency itself.[40] In our patient, serologic test results for collagen vascular disorders were negative, he was not taking any medications before becoming ill, and the appearance of his rash was not characteristic of adult-onset Still disease. Furthermore, meningitis as the initial manifestation of adult-onset Still disease is exceedingly rare, and, nearly 2 years later, our patient has not had any further evidence of arthritis.

Perhaps a bacterial etiology could not be identified because the organism was not present at the time of sampling or because the diagnostic tests used could not detect its presence. For pediatric patients with proven bacterial meningitis, blood culture detects the causative organism in 40% to 90%, CSF Gram stain in 60% to 90%, CSF culture in 70% to 85%, and CSF antigen testing in 50% to 100% of cases.[41] Polymerase chain reaction testing for specific meningeal pathogens[42–44] and for a broad range of bacterial pathogens[45] has also been used to determine the bacterial etiology of cases of meningitis. Several investigators[42–44] have shown that compared with CSF culture, polymerase chain reaction is a highly sensitive and specific means of diagnosing meningococcal meningitis. As more experience is gained with this diagnostic test, it will undoubtedly become a powerful tool in the diagnostic approach to patients with meningitis syndromes.

Although the CSF Gram stain is positive in 60% to 90% of cases of bacterial meningitis, results vary by organism and by the concentration of bacteria in the CSF. The CSF Gram stain is positive by organism as follows: *S pneumoniae,* 90%; *H influenzae,* 86%; *N meningitidis,* 75%; gram-negative bacilli, 50%; and *Listeria monocytogenes,* less than 50%.[46] Bacterial concentrations of 10^5 CFU/mL or greater are associated with positive CSF Gram staining in up to 97% of cases, whereas concentrations of 10^3 CFU/mL or less are associated with a positive Gram stain result in approximately 25% of cases.[47] There are limited data defining the CSF bacterial concentration in cases of meningitis due to *N meningitidis*. One study[48] of 9 patients with meningococcal meningitis found a mean concentration of 1.27×10^5 CFU/mL. In this small study, as few as 1,500/mL of meningococcal colonies were found (quantities clearly below the threshold of detection by Gram staining). Low CSF bacterial concentrations could explain why the Gram stain was negative in our patient, but this would not necessarily explain the negative culture findings.

The last potential reason why no bacterial cause was identified relates to the impact of antibiotic drug therapy on the results of diagnostic tests. Although the receipt of antibiotics before CSF sampling does not significantly alter the CSF WBC count, glucose level, or protein concentration,[49] such therapy can certainly affect blood culture and CSF Gram stain and culture results. In the presence of antibiotics, the ability to detect an organism by CSF Gram stain and culture drops to 40% to 60% and less than 50%, respectively,[50] and the rate of recovery from blood culture drops to less than 5%.[51] How fast antibiotics negate the ability to make a microbiologic diagnosis by CSF analysis is not known. A study of experimental pneumococcal meningitis in rabbits illustrates a 2-log drop in CSF bacterial concentration every hour after penicillin administration such that one would expect to see negative smears by 4 hours and sterilization by 8 hours.[52] This study is often quoted as evidence for a 4- to 8-hour window between antibiotic administration and LP before it is too late to make a microbiologic diagnosis. However, it is unclear whether these data can be extrapolated to infection due to organisms other than *S pneumoniae* or to humans.

In humans, the use of repeated LP illustrates that 90% to 100% of CSF cultures are sterile by 24 to 36 hours after the administration of appropriate antibiotics.[50] More precise human data on the rate of CSF sterilization have not been established, but in a recent retrospective study[53] of 128 cases of pediatric meningitis, the authors found that the interval between antibiotic drug administration and a negative CSF culture result ranged from 15 minutes to 60 hours. Three of 15 patients whose CSF was sampled within 1 hour of antibiotic drug administration had a negative CSF culture result, and the infecting organism for each of these 3 cases was *N meningitidis*. Furthermore, CSF sterilization for each of the 37 cases of meningococcal meningitis occurred within 3 hours, suggesting that CSF sterilization, in some cases, occurs faster than previously believed. Therefore, based on the existing literature, our patient's clinical presentation, and the fact that he is an LCCD host, we believe that he likely had meningococcal meningitis. Our inability to detect the organism may relate to 1 or more factors, including low CSF bacterial concentration and previous antibiotic medication exposure. His complement deficiency state itself may have contributed to an abnormal CSF formula (elevated protein level and neutrophilic pleocytosis) and low CSF bacterial concentration, but no data exist on the expected CSF findings in LCCD hosts with bacterial meningitis.

Once an LCCD individual is identified, the clinician should focus on education and counseling.[51] Patients and their

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families should learn how to recognize and respond to the early signs and symptoms of meningococcal sepsis and meningitis. Patients with a good understanding of their condition will likely be able to live a normal life without the overriding fear of recurrent meningococcal infection. A medical identification bracelet should be ordered for the patient, and appropriate family members should be screened to see whether they are unrecognized LCCD hosts.[55] Select patients (and their families) may benefit from meeting with a qualified genetic counselor.

Prophylactic strategies, including long-term chemoprophylaxis and immunoprophylaxis, should also be considered for LCCD hosts. Because complement-deficient hosts develop meningococcal infection relatively infrequently, and because the interval between recurrences may be many years, it is difficult to determine the efficacy of such prophylactic strategies. Antibiotic prophylaxis (ie, penicillin injected monthly) has been used successfully in this situation,[56,57] but there are several downsides to this approach, including difficulties associated with committing a child or young adult to lifelong therapy and the prospect of selecting out for antibiotic-resistant strains. Meningococcal vaccination in LCCD hosts has also been investigated by numerous researchers.[19–21,54,58–60] The tetravalent meningococcal vaccine promotes antibody production to capsular serogroups A, C, Y, and W-135, but LCCD patients must be educated that the vaccine lacks protection against serogroup B infection and fails to fully compensate for the absent complement-mediated serum bactericidal activity. Our patient was fitted for a medical identification bracelet and was administered the tetravalent meningococcal vaccine, and he and his family were educated about meningococcal disease and vaccine efficacy. To date, he has had no evidence of recurrent infection.

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D9

AN - NLM1411724
DT - Case Reports, Journal Article
TI - Recurrence of **neisserial meningococcemia** due to deficiency of terminal complement component.
AU - Morris J T; Kelly W J
PUB - Southern medical journal
- UNITED STATES
- Oct 1992
ORD - 1992-10-00
LNKD - **pubmed:1411724**
IRN - ISSN 0038-4348 *(Print)*
VOL - 85
NR - 10
PG - 1030 - 1031
LA - eng
IW - Adult; Bacteremia: diagnosis, drug therapy, etiology(#); Complement C6: deficiency(#); Humans; Immunologic Deficiency Syndromes: complications(#); Male; Meningococcal Infections: diagnosis, drug therapy, etiology (#); **Neisseria meningitidis**(#); Penicillin G: therapeutic use; Recurrence
AW - Complement C6; Penicillin G
AB - Recurrent infections with **Neisseria meningitidis** are attributed to deficiencies of terminal complement components. The serotype most commonly responsible for recurrent N meningitidis infections is serotype Y. We have reported a case of recurrent meningitis due to N meningitidis in a patient who was found to be deficient in the sixth component of complement. **Complement deficiencies** should be considered in any patient with recurrent infections caused by N meningitidis.

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AN - NLM15328683
DT - Case Reports, Journal Article, Review
TI - Complement C7 deficiency presenting as recurrent aseptic meningitis.
AU - Corvini Michael; Randolph Christopher; Aronin Steven I
PUB - Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology
- United States
- Aug 2004
ORD - 2004-08-00
LNKD - **pubmed:15328683**
IRN - ISSN 1081-1206 *(Print)*
VOL - 93
NR - 2
PG - 200 - 205
LA - eng
IW - Adult; Arthritis: diagnosis(#), etiology(#); Cerebrospinal Fluid: metabolism; Complement C7: deficiency(#); Complement Membrane Attack Complex: deficiency; Diagnosis, Differential; Humans; Immunologic Deficiency Syndromes: complications(#), diagnosis(#); Male; Meningitis, Aseptic: diagnosis(#), etiology(#); Recurrence
AW - Complement C7; Complement Membrane Attack Complex
AB - *BACKGROUND*
Complement deficiency states are rare inherited disorders that may predispose affected individuals to angioedema, collagen vascular disease, or infection due to encapsulated organisms, especially **Neisseria meningitidis**

- *OBJECTIVES*
To report the case of a 36-year-old man of Irish descent with recurrent culture-negative neutrophilic meningitis, to offer potential reasons for the inability to recover a causative pathogen, and to review the genetics and prevalence of **complement deficiency** states, the methods of screening for such deficiencies, the features of meningococcal infection as they relate to such deficiencies, and management strategies for clinicians caring for patients with such deficiencies.

- *METHODS*
The patient presented in 1988 and again in 2002 with culture-negative neutrophilic meningitis. His second episode was characterized by a rash suggestive of meningococcal infection, prompting immunologic evaluation.

- *RESULTS*
Immunologic evaluation revealed an undetectable CH50 level. Levels of C1, C2, and C5 through C9 were normal except for C7, which was undetectable. Further testing revealed that the patient's sister was also C7 deficient.

- *CONCLUSIONS*
Complement component deficiencies are relatively rare; individuals with collagen vascular disease and systemic neisserial infection should be screened using either the CH50 or the APH-50 assay. Key to the management of a late-complement component-deficient host is counseling, education about meningococcal infection, and discussions about the potential benefits of chemoprophylaxis and immunoprophylaxis. The ability to detect the bacterial cause of meningitis in such patients is organism dependent and may be influenced by factors such as cerebrospinal fluid bacterial concentration and previous antibiotic drug exposure.

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VACCINE AGAINST GROUP B NEISSERIA MENINGITIDIS: PROTECTION TRIAL AND MASS VACCINATION RESULTS IN CUBA

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SUMMARY

The Cuban vaccine, first in the world with proven efficacy against group B-caused disease, is based on outer membrane proteins from B meningococci capable of inducing long-lasting and high-titered bactericidal antibodies in humans. This bactericidal activity has a wide spectrum against all pathogenic group B *Neisseria meningitidis* tested.

A randomized, double-blind controlled trial of the vaccine efficacy was performed during 1987-1989 with 106 000 10-14 years old students from 197 boarding schools in seven provinces.

The efficacy obtained was 83% (χ^2, $p<0.002$; Fischer exact, $p<0.001$). In a second field trial including 133 600 persons from 5 months to 24 years of age in Ciego de Avila province (30 cases/10^5 inhabitants, the highest incidence rate in Cuba) by comparing vaccinated and non-vaccinated population after 2.5 years of observation and careful follow-up, the efficacy and safety was confirmed.

Because of these results and because of the very low reactogenicity of the vaccine, the Ministry of Public Health took the advice of the Scientific Council to vaccinate all children between 3 months and 6 years of age in the most affected provinces. No severe or long lasting reactions to the vaccine were observed after the millions of doses administered. The efficacy of vaccination varied in the provinces between 83% and 94%, among age groups ranging from 3 months and 20 years.

After 3 years of massive application no severe reactions occurred and one of the most severe epidemics has been practically eradicated.

Key words: Evaluation studies; Neisseria meningitidis; Randomized controlled trials; Vaccines

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INTRODUCTION

Since 1976, meningococcal disease constitutes a serious health problem in Cuba due to the significant rise in incidence of this disease and its high lethality.

The causal agents at beginning of the outbreak were predominantly group C meningococci and in second place group B meningococci; after the mass vaccination campaign conducted in 1979 using the polysaccharide Merieux A + C, group C caused meningococcal disease was practically eradicated.

In the late 70s and early 80s, a significant incidence increase took place, virtually caused by B meningococci of 4:P1.15 (95%), 15:P1.15 (3%) and 15:P1.16 (1%) serotypes and subtypes.

The peak epidemic year was 1983, although the situation differed from province to province. Higher incidence rates were observed in 1985 in children younger than 6 years (50 cases per 10^5 inhabitants) and among teenagers (20 cases per 10^5). The most affected teenagers were those attending secondary boarding schools in the central provinces of Cuba (57,7 cases per 10^5 students).

B-meningococcal disease has been the most serious infection health hazard of the last two decades in Cuba.

Repeated testing with different vaccine candicates in the United States, Europe, the Soviet Union, and South Africa, revealed many deficiencies among these; a weak, very variable and short-lasting (5-7 months) bactericidal activity; beyond 5-7 months after immunization the concentration of specific antibodies of IgG class, directed against important exposed proteins and other antigens of the bacterial envelope, was very low. In addition, weak or absent anamnestic response to a booster dose after 8 to 12 months of primary immunization was observed as one of the most critical failures.

Thus, taking into consideration the world research state in the B meningococcus vaccine development and our situation with the disease, decision was taken to obtain in short time a good candidate vaccine.

Among different vaccine formulations one was selected for which carefully conducted preclinical and phase I and phase II clinical studies revealed innocuousness, safety and very low reactogenicity, long-lasting bactericidal and specific antibody titers of a broad spectrum against different pathogenic serotypes and subtypes. On the basis of these promising serological efficacy results, this vaccine has been used in a double-blind placebo-vaccine field trials for epidemiological efficacy evaluation.

MATERIALS AND METHODS

Base-line studies

Base-line data on epidemiology of the disease and diagnostic laboratory capability were collected and evaluated during a three-year period in the provinces, before the trial-started (1984-87). Laboratory procedures, case management protocols, and the system of coordinating information from schools, hospitals, provincial, municipal and national epidemiologists, and laboratories have been carefully reviewed, evaluated and when needed established.

Case ascertainment and case definitions were established as well as the isolates and samples transport-conservation-study system.

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Vaccine trial design

The trial was a randomized, placebo-controlled, double blind trial of vaccine efficacy. The protection trial was performed among teen-agers attending secondary boarding schools. The target group ranged, from 11 to 16 years of age and was distributed in 197 schools and 7 provinces with the highest incidence rates in this age group (40 cases per 10^5 students at the starting point of the trial).

Sample size was calculated according to the level of efficacy to be detected at 75% or greater, with less than a 0.05 type I error ($\alpha = 0.05$) and a power of 80% ($\beta = 0.2$, taking into account an attack rate of the disease of 40 cases per 10^5 students and a follow-up period of 1-3 year. With this incidence rate at the start of the trial and considering the other statistical factors, 106 251 students for placebo and vaccine groups were calculated to be needed in using schools as randomization units. Each school with approx. 540 students constitutes a homogeneous unit, either completely vaccinated or completely placebo inoculated.

A list of schools was assembled and stratified by province according to incidence of the disease during the last three years, to assure homogeneous distribution of exposure to risk of disease in receiving placebo or vaccine. Afterwards, placebo or vaccine were randomly allocated in each school within every stratus.

To assure blinding, lot identification was performed with randomly generated numbers having a secret master code list. Ten different numbers for placebo and ten for vaccine were used.

The vaccine

The vaccine components are purified proteins from the outer-membrane of group B meningococci enriched with proteins from the high molecular weight proteins complex (65-95 KD) having the greatest capacity of inducing long-lasting bactericidal antibodies. Some of the purified proteins, together with a good controlled proportion of phospholipide and lipopolysaccharide (LPS) form a proteoliposom, and the added amounts of High Molecular + Protein-Complex is non covalently conjugated or attached to the proteoliposome surface. In addition, the whole protein complex is conjugated non covalently, 1:1 proportion with C polysaccharide and finally the whole complex is adsorbed to aluminium hydroxide gel with a controlled particle size.

Vaccine formulation

Each dose contains:

Outer membrane proteins from B meningococci	50 mcg
Purified polysaccharide form C meningococci	50 mcg
Aluminium hydroxide gel	2 mg
Sodium thiomersalate	0.01 mg
PBS csp	0.5 ml
LPS	1%

The commercial name of this preparation is VA-MENGOC-BC^R, a trade mark of Institute Finlay, Habana, Cuba.

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Control vaccine (Placebo)

The control vaccine was identical in appearance to the tested vaccine and consisted of a saline placebo containing the adjuvant aluminium hydroxide.

Vaccination, sampling and evaluation

A two 0.5 ml deltoid intramuscular dose with 6 to 8 weeks interval was the vaccination schedule established.

In each province, 300 students randomly chosen were used for immunogenicity and carrier state studies. The samples for these studies were taken immediately before applying the first dose and 4 weeks after the second one. Additional blood and exudate samples were taken every six months after vaccination. ELISA and bactericidal studies were performed as described elsewhere (1).

Only cases with the following criteria were used for efficacy evaluation: confirmed cases are only those with clinical illness from which a B meningococcus was isolated by culture or those with clinical illness with Gram-negative diplococci directly observed and where a latex or ELISA confirmed B antigen was found.

The standard forms for data collection, previously field-tested, were submitted to the Ministry of Public Health and stored in the Efficacy Trial Data Base Computer System.

SAS-PC version 6.03 and EPI-INFO were used for data analysis and study monitoring, relative risk calculation for vaccine and placebo groups with schools as randomization units, individual or cluster approaches. The comparisons between placebo and vaccinees regarding ELISA and bactericidal titer distributions were performed with the help of a computing program having common non-parametric tests, Wilcoxon Rank-Sum Test, t-Test, percentile distribution and binomial distribution analysis of probabilities and the statistical analysis significance tests-Chi-square and Fischer Exact.

RESULTS AND DISCUSSION

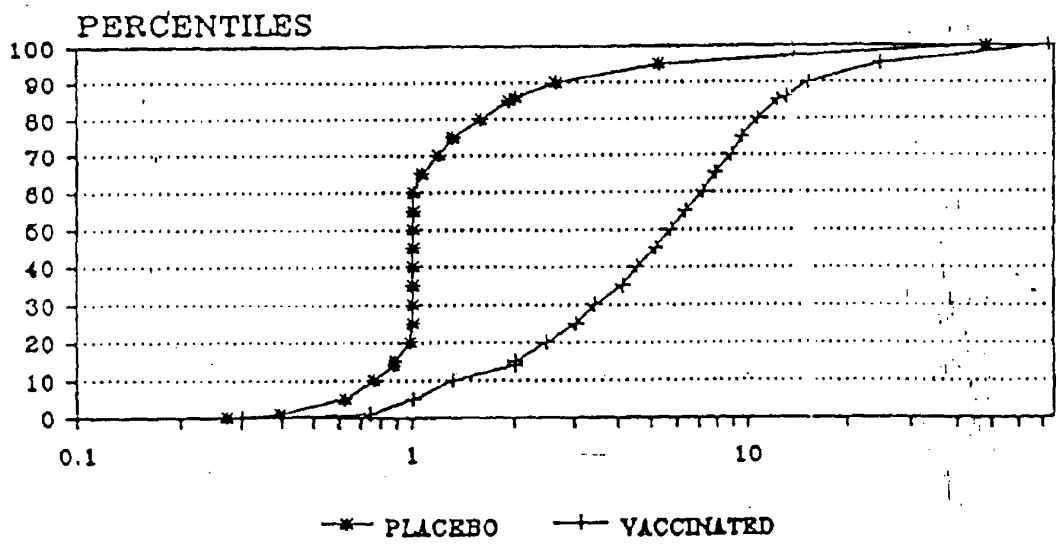


Figure 1
Cumulative distribution of the ratio ELISA2/ELISA1 four weeks after vaccination

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The study of the meningococcus carrier state shows no significant difference between placebo-injected controls and vaccinees when the sample was taken four weeks after vaccination. Six months after vaccination, a significant lowering in carrier state in vaccinees was observed, but more careful assessment of variations and mechanisms must be performed in the future for conclusive statements.

In the vaccine group, an 88% of seroconversion (E2/E1) was detected using the ELISA method; in percentile 50 (median of the group), a six-fold increase was observed. On the other hand, in the placebo group, percentile 80 shows just one as E2/E1 ratio and only 14% of this group shows two-fold increase (correlating quite well with carrier state). Comparing the cumulative distribution of the E2/E1 ratio in placebo and vaccine four weeks after vaccination, a significant difference with $p < 0.001$ is seen..(Fig. 1).

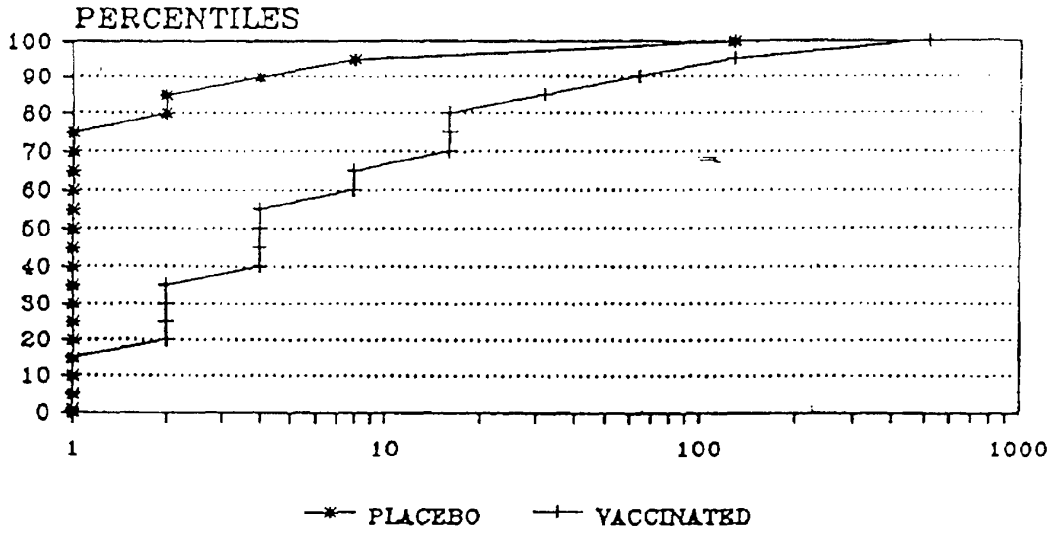


Figure 2
Cumulative distribution of the ratio BACTE2.Titer/BACTE1.Titer four weeks after vaccination

Seroconversion regarding bactericidal activity was detected in 85% of the vaccine group in which percentil 50 (median) shows a four-fold titer increase (Fig. 2). The difference with respect to the placebo group was highly significant, $p<0.001$. Only percentil 85 shows two-fold increase in this group caused by normal contacts with meningococci in the population. The cumulative distribution of the ratio B2/B1 comparing placebo and vaccine group shows statistical significance.

This study lasted 16 months, during which 25 culture and latex positive confirmed cases occurred in the study population, four in the vaccine group and 21 in the placebo group. The timing of cumulative case occurrence among placebo and vaccine group, as seen further on, constitutes a good graphic demonstration of the vaccine effect (Fig.3). Case occurrence was distributed per school as indenpendent cases and fulfilling the criteria of «case definition» needed in order to be included in the efficacy calculation.

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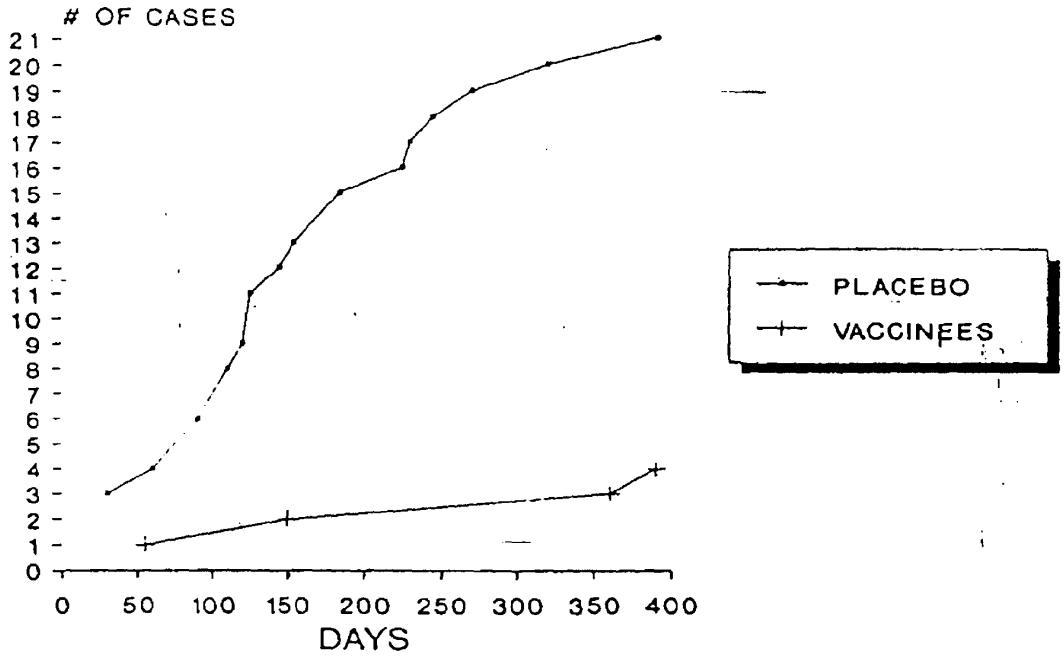


Figure 3
Timing of cumulative case occurrence

Further observation up to 24 months revealed the occurrence of five more bacteriologically confirmed cases, all in the placebo group and none among the vaccinees though not included in the following efficacy calculation:

Efficacy according to conglomerate approach

	Vaccine	Placebo	Total
Total of schools involved	99	98	197
Schools with cases	3	17	20
Total of cases	4	21	25

Analysis of Single Table
Relative risk-0.17 (0.05<RR <0.58)
Taylor series 95% confidence limits for RR

	Chi-squares	p-values
Uncorrected	11.07	0.0008791
Mantel-Haenszel	11.01	0.0009061
Yates corrected	9.55	0.0019968

Resultant efficacy: 83%
Confidence interval: 95-42%

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Efficacy according to individual approach

Group	Cases	Not ill	Total
Vaccinee	4	52962	52966
Placebo	21	53264	53285
Total	25	106226	106251

Analysis of Single Table
Relative risk-0.19 (0.07< RR<0.58)
Taylor series 95% confidence limits for RR

	Chi-squares	p-values
Uncorrected	11.46	0.0007107
Mantel-Haenszel	11.46	0.0007108
Yates corrected	10.15	0.0014457

Resultant efficacy: 81%
Confidence interval: 93-44%

Vaccine impact is also shown by demonstrating the significant reduction in case-occurrence probability in vaccine schools in comparison with placebo treated schools. Immediately before the starting point of the field trial the probability of one or more cases per school in the study was calculated (Po = 0,192). After 1.3 year of follow-up period 17 cases had occurred in 98 placebo schools. The probability of case occurrence was 0.173469 (Pp). The simultaneous case occurrence in vaccine schools was 3 in 99 schools.

Binomial distribution

	N = 99	P.1734693
X	P(X)	Cumulative probability
3	.00001	.00001
4	.00005	.00006
5	.00019	.00025
6	.00062	.00086
E(X) = 17.173346	std dev = 3.76754	variance = 14.19439

A way to evaluate the impact of the vaccine is by calculating under the hypothesis that Po = 0.192 (previous value) the probability of the happening in placebo and vaccine groups. The probability associated with the occurrence of three or less schools with at least one case in a set of 99 (vaccine groups) and 17 or less of a set of 98 (placebo groups) are 0.00001 and 0.38 respectively. These results suggest that p in the vaccine group is

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significantly less than Po, but not so in the placebo groups. Another approach is using the placebo value of p in determining the probability of that happening in the vaccine group. Having p the value of 0.173 (17/98), then, P (X3)0.00001. This value of p is significantly less than 0.173.

The difference in case occurrence among placebo and vaccine groups was statistically significant, considering the cases individually or effecting a conglomerate (school distribution of cases). The vaccine proved to be effective in the study population in 81% (individual treatment of cases) or 83% (conglomerate treatment), and the confidence interval from 44 to 93% or 42 to 95% respectively.

Further follow-up studies

Furthermore, a good correlation between serological evolution and protection as well as the low reactogenicity and safety of the vaccine was confirmed.

The comparison of ELISA and bactericidal ratios E4/E1 and B4/B1 respectively constitutes an objective proof of duration of the vaccine protective capacity. E4 and B4 represent ELISA and bactericidal values one year after vaccination (Figs. 4 & 5).

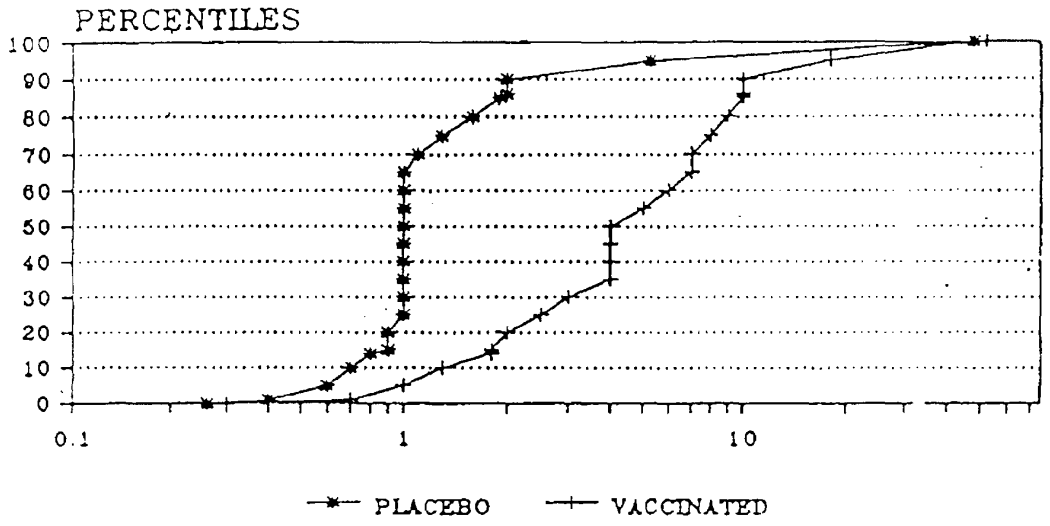


Figure 4
Cumulative distribution of the ratio ELISA4/ELISA1 one year after vaccination

The anamnestic antibody response was studied in army recruits, in parallel. A booster dose was tested in one group after one year and another group after 18 months. Before boostering (12 to 18 months) blood plasma from the vaccine group of the parallel study was collected, and the IgG fraction was purified and tested by ELISA and bactericidal activity (Figs. 6 and 7).

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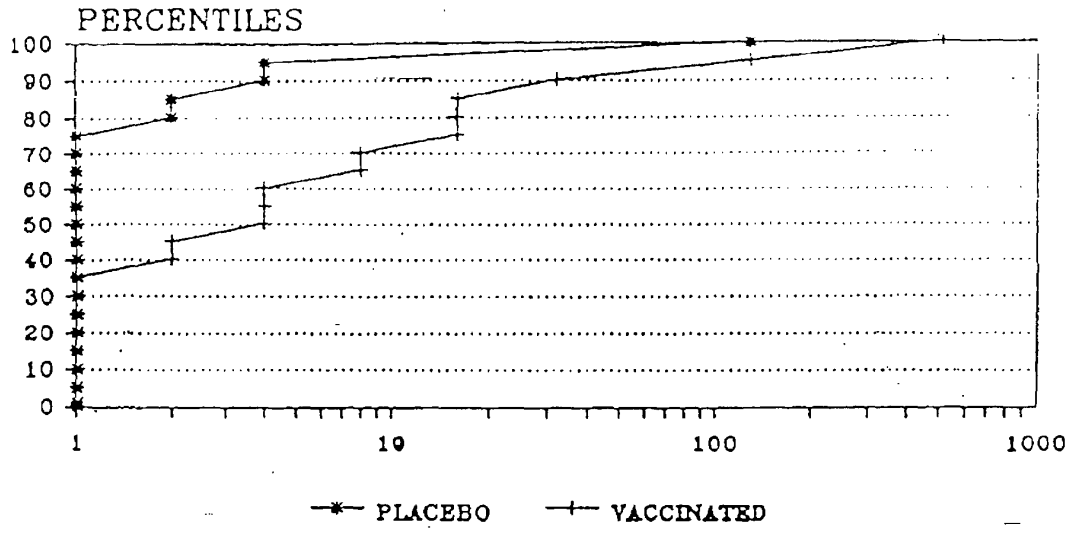


Figure 5
Cumulative distribution of the ratio BACTE4.Titer/BACTE1.Titer one year after vaccination

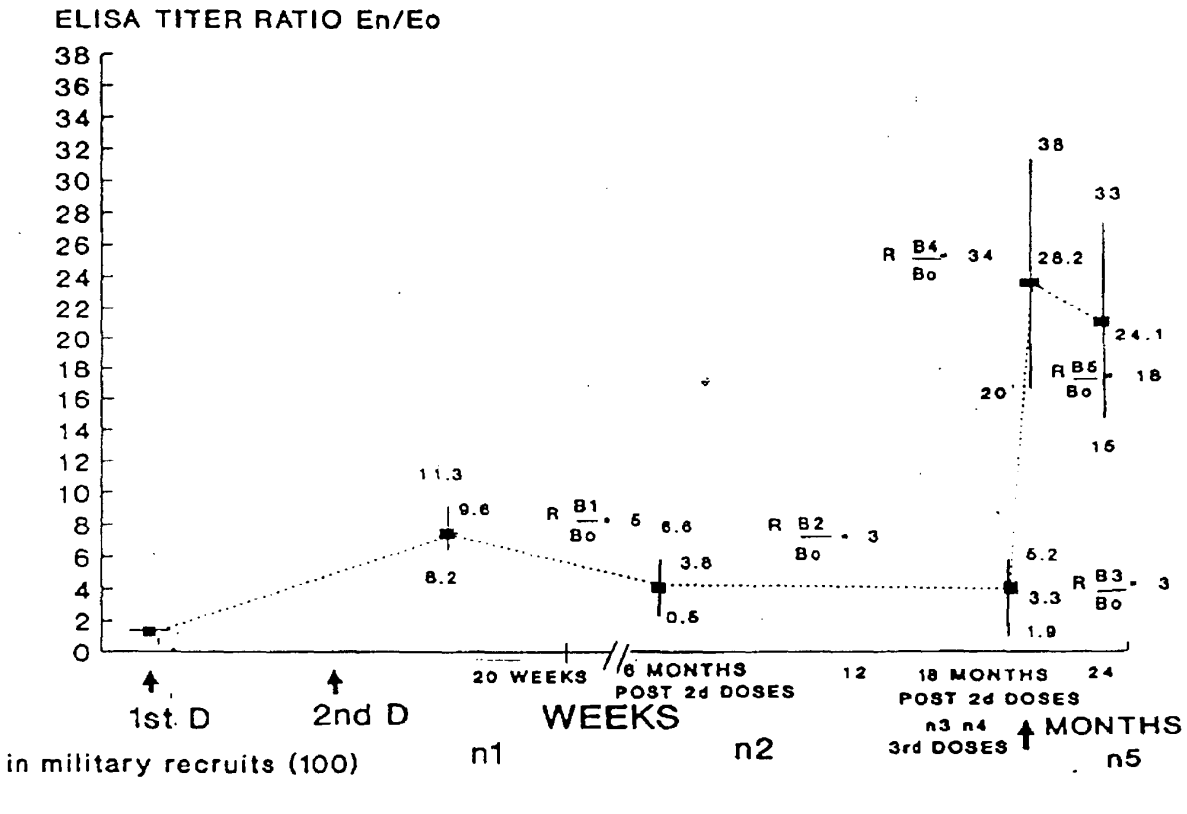


Figure 6
Increasing bactericidal and specific antibodies with a booster

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The results of these tests indicated long-lasting bactericidal and specific antibodies against some of the most frequent pathogenic B serotypes. After boostering the specific antibody increased by more than 5 times and the bactericidal titer increased more than 10 times, showing an excellent anamnestic specific response.

Summary of mass vaccination results

Taking into consideration the high incidence of meningococcal disease among children up to six years of age occurring in Cuba and the satisfactory reactogenicity and efficacy studies with the Antimeningococcal BC Vaccine «VA-MENGOC-BC®» evaluated during the last years, a mass vaccination campaign was organized, starting with this age-group (1989-90). Table 1 shows the percentage of vaccination per province in the age-group below six years.

An independent group of the Tropical Medicine Institute «Pedro Kouri» of Ministry of Public Health evaluated in a carefully conducted study, the effectivity of the mass vaccination campaign. Table 2 shows the summary of efficacy, in general reaching 92.5%.

One very clear example of the vaccine impact was observed in the province Havana City, Fig. 8, where the incidence rate among children below six years rose constantly (1986 - 27.5/10^5, 1987 - 30/10^5, 1988 - 42.5/10^5) and after the vaccination the strong epidemic wave was stopped (1989 - 15/10^5, 1990 - 3.8/10^5, 1991 - 1/10^5) and among vaccinees less than 0.08/10^5).

Actually in Cuba the population between 3 months and 20 years is vaccinated; the VA-MENGOC-BC® vaccine is incorporated to the infancy vaccine scheme (for 3 months children).

Also the vaccination of other risk-groups is practically concluded; elderly homes, military camps, big schools, industries, *etc.*

In a very short time the meningococcal disease will be completely controlled.

Reference

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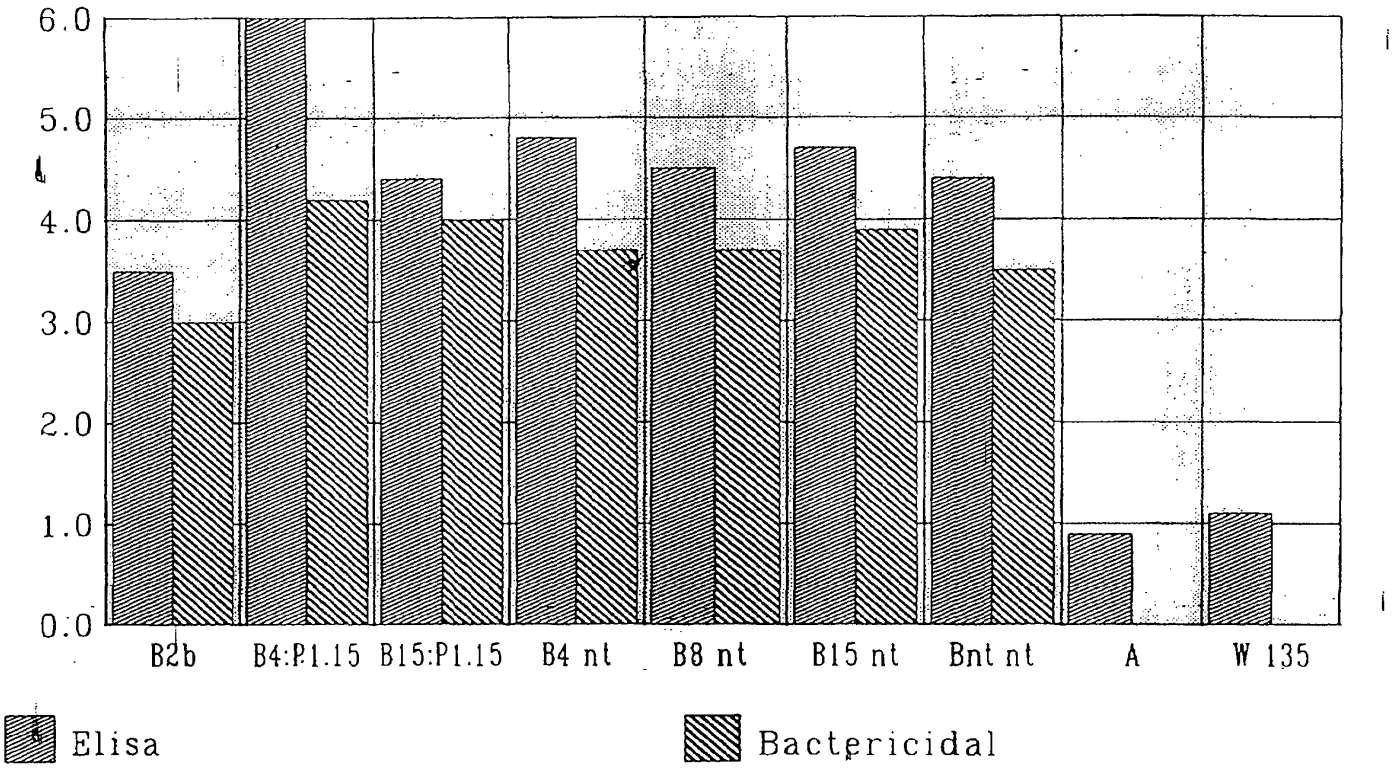


Figure 7
Specific and bactericidal antibody titer increases maintained up to 18 months after vaccination in military recruits against different pathogenic serotypes

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Table 1
Percentage of vaccination per province in age group < 6 years

Province	Population	Vaccinees	%
PR	67 500	56 623	83.8
CH	182 400	161 670	88.6
MT	53 000	48 815	92.1
VC	70 000	61 047	87.2
CF	33 800	32 094	95.0
SS	37 700	31 935	84.7
CM	73 500	61 917	84.2
LT	53 100	43 808	82.5
HO	102 900	91 191	88.6
GM	94 800	72 898	76.9
SC	116 600	100 820	86.5
GT	61 100	53 679	87.9
TOTAL	**1 013 900**	**866 148**	**85.4**
Source: Division of Epidemiology, Ministry of Public Health			

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Table 2
Relative risk and efficacy per province in age group < 6 years

Province	Relative Risk	Efficacy %
PR	9.37	89.3
CH	39.06	97.44
MT	23.33	88.57
VC	24.55	95.90
CF	23.00	95.68
SS	11.08	90.97
CM	7.48	86.60
LT	23.60	89.28
HO	97.49	98.97
GM	29.99	96.66
SC	6.84	85.47
GT	18.11	94.47
TOTAL	13.44	92.56

Source: Tropical Medicine Institute «PEDRO KOURI», Ministry of Public Health

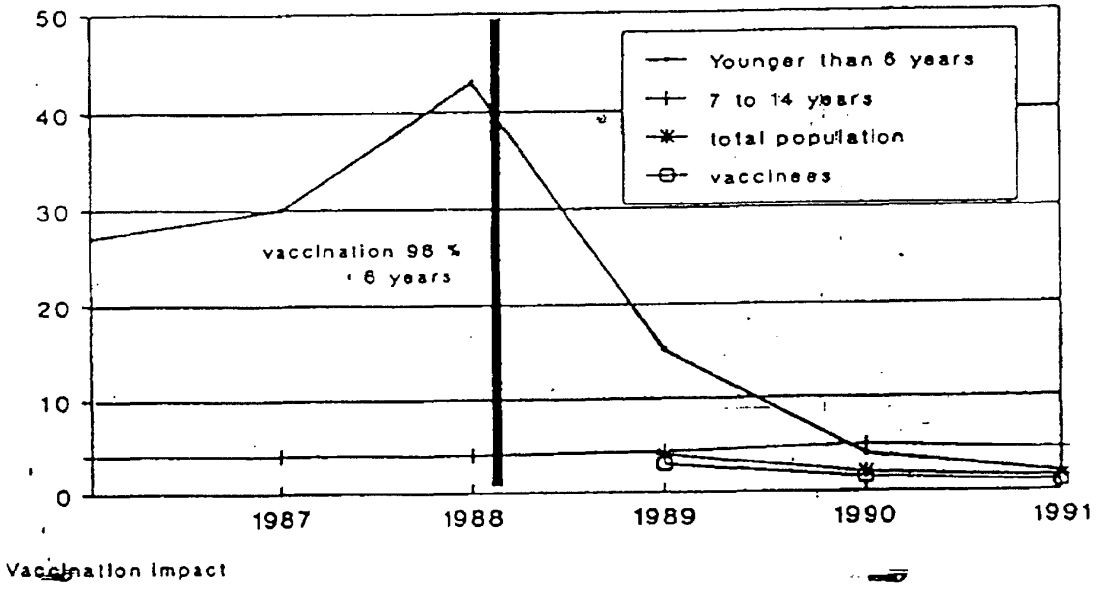


Figure 8
Meningococcal Disease in Havana city. indicence rate per 100 000

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DISCUSSION

Gotschlich:
To what do you attribute the broad specificity of the vaccine? What strains were tested for bactericidal effect, and what is the relative bactericidal effect against other strains?

Sierra:
All the results are related to the vaccine strain B:4:P1.15. We have also evaluated the spectrum of specific antibodies in ELISA and in bactericidal assay using different strains.

Gotschlich:
Could you just give us a list of the strains against which the bactericidal test was performed?

Sierra:
We have used B:4:P1.15, B:15:P1.15, B:4:NT, B:2a, B:2b, B:2c, B:8.

Gotschlich:
Could you try to speculate on the reason for the wide cross reactivity; which antigens do you think this is due to?

Sierra:
We think that this long lasting cross reactivity is due to the presence of high molecular weight proteins. There are six important proteins in this rank between 65 to 95 000 daltons. Two or three of these are iron-regulated proteins.

Gotschlich:
Which ones are iron-regulated?

Sierra:
One is a protein of 80 000 daltons, the other is very near to 90 000 daltons. We have now cloned, expressed, and purified some of these high molecular weight proteins, and we have characterized them. It is very difficult to obtain these in sufficient amount for characterization. We have demonstrated the importance of these proteins, especially for the immunological memory and for the cross reactivity.

Gotschlich:
There was yesterday a remarkably careful follow-up of patients that had been vaccinated, in terms of long term sequelae. Have you had the opportunity to investigate this in the same details as what was presented yesterday?

Sierra:
Yes, we studied it in Cuba very carefully. The first group of people was vaccinated in Cuba in 1985, and we have until now followed all the groups of the phase 1, phase 2, and phase 3 trials. We have no long-lasting sequelae at all. We have no disease caused and established through the vaccine. The most important reactions we have observed are the purpuric reactions. Very few cases have occurred, all over the country, especially in very small children. They were very short-lasting reactions. There were no sequelae and no neurologic diseases at all.

Peltola:
What do you mean by purpuric reactions? Do you mean petecchiae? If so, they usually are not very short-lived.

Sierra:
There are two types of reactions. One type of rash seems to be some type of allergy to some of the components and the other type of reaction consists of very small petecchiae. Both are short lasting. I think it is very important also to take into consideration the experience that we have in collaboration with Brazil in São Paulo. They have vaccinated nearly two and a half million children below six-seven years of age within in one week. They have experience in evaluating these side reactions.
They also observed a small number of these purpuric reactions or petecchiae. As far as I know, in Cuba or in Brazil we don't have long-lasting sequelae disease or severe reactions caused through the vaccine.

Griffiths:
You think that these iron-regulated proteins are important in your vaccine. Do you take any special measures to insure that they are expressed?

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Sierra:
I have not said that the iron regulated proteins are the only ones responsible for the efficacy. I have said that among the four most important high molecular weight proteins responsible for the long lasting bactericidal response, at least two proteins were related to iron metabolism.

Gotschlich:
The question was: Do you purposely limit iron for growth?

Sierra:
We have performed some changes in media regarding the concentration of some nutrients, but not especially iron limitation.

Wetzler:
Were there any non-volunteers in your study, and if there were, what was the incidence of disease in that group?

Sierra:
The incidence among people not volunteering in the study, in the same region or in the neighbourhood of these schools, has remained on the same level. In that age group we have studied this question intensively. We are going to publish all these results very soon.

Robbins:
I have two questions. The dose of the vaccine you mentioned was 50 micrograms. Was that based upon protein? And if it was based upon the protein, what was the total weight of all the material in the vaccine per dose? And the second question: what percentage of cases of bacterial meningitis were you unable to diagnose either by culture or by serological methods?

Sierra:
The vaccine composition is 50 micrograms of proteins per dose. Between 12 and 18% of these are proteins of the high molecular weight complex. We purify the outer membrane proteins, purify the proteoliposomes and we enrich these preparations with high molecular weight proteins. This is not simply a purification route. The composition is approx. 85% of the normal serotype proteins and approx. 15% of high molecular weight proteins. And we try to avoid the presence of class 4 and class 5 proteins in the vaccine because we have found a great variability in the presence and expression of these proteins in the strains circulating in our country, even before the P1.15 epidemic. We have a limit for tolerance of these proteins in the vaccine. We also avoid the B polysaccharide in the vaccine. We test the preparation chemically and also for the capacity to induce antibodies. We have found that the preparation is not able to induce significant anti-B antibodies.
To the second question, related to the incidence: we were not able to find the causal agent in three cases among vaccinees and in two cases of placebo. But after these we have applied different serological analytical methods. We have tested very carefully for the presence of antigens.
A case of meningococcemia in a vaccinee in the Olgin province was excluded because through culture, latex and ELISA methods we were not able to find B antigen.

Broome:
I would like to look a little further into these excluded cases. There were clinically diagnosed two cases of meningococcemia, one fatal in vaccinees and one also fatal in the placebo recipients. They certainly did not have culture confimation, so they did not meet your primary case definition. But in adolescents vaccinated against group C experiencing a group B epidemic, I think those cases are of some consideration as potential group B cases. My expericence with group B antigen testing is that is is not particularly sensitive, certainly not as sensitive as group A or group B antigen detection. I wonder if you could tell us what is the LPS content of the vaccine.

Sierra:
The LPS content of the vaccine is 1% of the total protein amount. If we include the cases without culture the efficacy is around 75%. In one of the three cases mentioned we had not demonstrated B antigens but we have demonstrated other bacterial antigens

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Gotschlich:
You attribute a high importance to the high molecular weight proteins that you add to you vaccine. Could you outline for us the principles of how you obtain these?

Sierra:
We have found one strain with very important growth characteristics in our culture media. We limit in our culture media some of the nutrients, but not iron especially. We are thinking also of doing that in the future. We found that this strain is able to produce higher concentrations of high molecular weight proteins than the other strains. We perform very difficult steps in purification. We purify these high molecular weight proteins, with successive chromatographic steps, and precipitation, and concentration steps. Then, we use them to enrich the preparation of vesicles.

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ADIS R&D PROFILE

Drugs R D 2007; 8 (1): 61-68
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Eculizumab

5G1.1, h5G1.1, Long-Acting Anti-C5 Monoclonal Antibody
5G1-1, Long-Acting Anti-C5 Monoclonal Antibody 5G1.1

Abstract

Eculizumab [*long-acting anti-C5 monoclonal antibody 5G1-1, 5G1.1, h5G1.1, monoclonal antibody 5G1.1, Soliris™*] is a monoclonal antibody that binds to the C5 complement molecule, thereby blocking the progression of the complement cascade at this point. By binding to C5, eculizumab prevents generation of the potent anaphylatoxin C5a and the cytolytic C5b-9 complex, or membrane attack complex. The compound has been filed for approval in the US and the EU for paroxysmal nocturnal haemoglobinuria (PNH), a chronic blood disease caused by a defective enzyme that leads to a lack of protective natural complement inhibitors. It is also being investigated for the treatment of asthma and transplantation.

Eculizumab is a long-acting, humanised version of the anti-C5 antibody [*h5G1.1*]. The short-acting version is called pexelizumab.

In January 2003 Alexion Pharmaceuticals executed a large-scale product supply agreement with Lonza Biologics for the long-term commercial manufacture of eculizumab. Specific terms of the agreement were not disclosed.

Alexion signed an agreement with GTC Biotherapeutics in 2000 for the production of eculizumab in transgenic goats. However, this agreement no longer appears to be active.

Alexion has also filed for approval for eculizumab in PNH in the EU under the centralised procedure. The EMEA has granted Alexion's request for evaluation of the proposed MAA for eculizumab in PNH under the accelerated assessment procedure. This shortens the review part of the MAA procedure.[1,2]

Results from the phase III TRIUMPH trial have been reported; all prespecified primary and secondary endpoints were achieved with statistical significance.[3]

The last patient completed the last visit in the SHEPARD trial in October 2006, and 12-month data is being submitted to regulatory applications. Positive preliminary interim results of 6 months of therapy have been reported. The study has been conducted to collect additional data on eculizumab's safety among haemolytic PNH patients who have undergone transfusion. The trial enrolled 97 patients in the US, Canada, Europe and Australia and includes 12 months of treatment.[4,5]

A pilot, open-label phase Ib trial evaluating eculizumab in 11 patients with PNH has been completed in the UK. All 11 patients in the trial chose to participate in two consecutive 1-year extension studies. One of these is still ongoing, with patients having completed at least 26 months of chronic eculizumab therapy. One- and two-year cumulative results have been presented. The positive findings further supported progression of the clinical development of eculizumab for PNH.[6,7]

Eculizumab has been granted orphan drug status in the US and EU for the treatment of PNH.[8].

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Phase I and II trials in rheumatoid arthritis had previously been completed.

The rationale behind evaluating eculizumab in this indication was that activated terminal complement proteins appear to have a central role in the pathogenesis of immune-mediated joint inflammation, indicating that anti-C5 monoclonal antibodies may have therapeutic potential for the treatment of rheumatoid arthritis.[9]

A phase I pilot trial was completed in patients with dermatomyositis, an inflammatory skin and muscle disorder, for which eculizumab has orphan drug status in the US.

The US FDA granted eculizumab fast-track designation in membranous glomerulonephritis in early 2000 and the drug has orphan drug status in the US for idiopathic membranous glomerular nephropathy.

Both long-acting and short-acting versions of anti-C5 monoclonal antibody 5G1.1 are being developed by Alexion Pharmaceuticals in the US, with the use of technology licensed from Stanford University. Eculizumab is covered by US Patent No. 6 355 245, which extends to C5-specific monoclonal antibodies for the treatment of inflammatory diseases.

The Japanese Patent Office issued patent number 3 734 266 for eculizumab in Japan in February 2006. The patent includes the use of eculizumab to inhibit complement activation in humans, the key mechanism of action used for the treatment of PNH. Corresponding patents have been issued or are pending in Europe, Canada, Australia and other key markets.[10]

1. Profile

1.1 Adverse Events

Eculizumab was safe and well tolerated without any cases of antibodies against the drug detected among 11 patients with paroxysmal nocturnal haemoglobinuria (PNH) in a phase Ib trial. The most common adverse events were headache, upper respiratory infection, sore throat, muscle/joint aches and influenza-like symptoms. Severe adverse events included viral chest infection, dizziness and shivering. Adverse events were similar in type and frequency to those reported with eculizumab or placebo in other controlled trials.[6,11,12] Two-year cumulative results from this study showed that eculizumab continued to be well tolerated. There have been four serious adverse events but no patients have discontinued treatment because of adverse events.[7]

In a multicentre, double-blind, placebo-controlled, ascending-dose trial, 40 patients with rheumatoid arthritis received a single dose of the antibody eculizumab. Within the dose range tested (0.1–8.0 mg/kg), eculizumab was safe and well tolerated. It had no detectable immunogenicity.[13]

Treatment with eculizumab for 2 months was safe and generally well tolerated in a phase I pilot trial in 40 patients with psoriasis. Adverse events included headache and nonspecific pain. 40% of placebo recipients and 10% of the treatment group withdrew from the study before completion.[14]

In a phase I pilot trial, 13 patients with dermatomyositis were randomised to receive eculizumab or placebo. Eculizumab was administered intravenously at a dose of 8 mg/kg once a week for 5 weeks and then once every 2 weeks for up to 2 months. Adverse events were similar in drug-treated and placebo-treated groups. The most common adverse events were rash and headache.[15]

In the double-blind, C99-004 trial, 117 patients with membranous nephritis were randomised to re-

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Table I. Features and properties

CAS number	219685-50-4
WHO ATC code	D05 (Antipsoriatics) L04A (Immunosuppressive Agents) B06 (Other Hematological Agents) G04 (Urologicals) L04A-A (Selective immunosuppressive agents) M01 (Antiinflammatory and Antirheumatic Products) R03 (Drugs for Obstructive Airway Diseases) R07A (Other Respiratory System Products)
EphMRA ATC code	R3 (Anti-Asthma and COPD Products) R7 (Other Respiratory System Products) B6 (All Other Haematological Agents) M1 (Anti-Inflammatory and Anti-Rheumatic Products) G4 (Urologicals) D5 (Nonsteroidal Products for Inflammatory Skin Disorders) L4A (Immunosuppressive Agents)
Originator	Alexion Pharmaceuticals: USA
Highest development phase	Phase Preclinical (USA)
Properties	
Mechanism of action	Complement factor 5A inhibitors
Pharmacodynamics	Prolongs cardiac xenograft survival in an *ex vivo* model; protects red blood cells from activated complement proteins
Route	Unknown route
Adverse events	Occasional: Back pain, Headache, Joint disorders, Muscle pain, Nasopharyngitis, Nausea, Pain, Respiratory tract infections

ceive placebo, eculizumab 8 mg/kg every 4 weeks or eculizumab 8 mg/kg every 2 weeks for 4 months. Eculizumab appeared to be well tolerated, with the most common adverse events being upper respiratory tract infection, diarrhoea and headache.[16]

In the E99-004 trial, 72 placebo and eculizumab patients with membranous nephritis from trial C99-004 were entered into a pre-planned, open-label extension study designed to provide eculizumab 8 mg/kg every 2 weeks for 12 months. The trial is still ongoing and current results show that eculizumab appears to be well tolerated with the most common adverse events being upper respiratory infections, nasal congestion/rhinitis and headache.[16]

In a double-blind, randomised, placebo-controlled phase IIb trial in approximately 350 patients with chronic rheumatoid arthritis undergoing treatment with methotrexate or leflunomide, eculizumab was administered either once per month or biweekly for 6 months. The rates of both serious and common adverse events were similar for placebo and eculizumab. The most common adverse events were upper respiratory tract infections, headache and nausea. Serious adverse events included myocardial infarction, accidental injury and cerebral infarction.[17]

Eculizumab had an adverse event profile comparable to placebo in a trial in 87 patients with paroxysmal nocturnal haemoglobinuria (the TRIUMPH trial). The most frequent adverse events with eculizumab were headache, nasopharyngitis and back pain.[3]

Interim 6-month results from the SHEPHERD trial showed that eculizumab appeared to be safe and well tolerated, with the most frequent adverse events being headache, nasopharyngitis and nausea.[5]

1.2 Pharmacodynamics

1.2.1 Genitourinary Disorders

Mechanism of action: Eculizumab appears to inhibit the production of activated terminal complement proteins, which destroy PNH red blood cells that lack complement protection.[12]

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Table II. Drug development history

Nov 1995	Preclinical development for Rheumatic disorders in the US (Unknown route)
Feb 1996	Preclinical development for Transplant rejection in the US (Unknown route)
Feb 1996	Preclinical development for Adult respiratory distress syndrome in the US (Unknown route)
Aug 1996	Preclinical development for Lupus nephritis in the US (Unknown route)
Jan 1998	IND filed in the US for clinical testing of h5G1.1 in rheumatoid arthritis
May 1998	Preclinical development for Systemic lupus erythematosus in the US (Unknown route)
Aug 1998	Phase-II for Systemic lupus erythematosus in the US (Unknown route)
Aug 1998	Phase-II for Rheumatoid arthritis in the US (Unknown route)
Apr 1999	Alexion has completed a phase I/II trial in rheumatoid arthritis in the US
Jul 1999	No-Development-Reported for Xenotransplant rejection in the US (Unknown route)
Jul 1999	No-Development-Reported for Adult respiratory distress syndrome in the US (Unknown route)
Aug 1999	Phase-I for Lupus nephritis in the US (Unknown route)
Oct 1999	Phase-II for Glomerulonephritis in the US (Unknown route)
Jan 2000	Preclinical development for Psoriasis in the US (Unknown route)
Feb 2000	Preclinical development for Dermatomyositis in the US (Unknown route)
Mar 2000	Eculizumab has received fast-track status for glomerulonephritis in the US
Mar 2000	Phase-II for Lupus nephritis in the US (Unknown route)
Apr 2000	Preclinical development for Bullous pemphigoid in the US (Unknown route)
Aug 2000	Patient enrolment completed in a phase II efficacy trial of h5G1.1 in rheumatoid arthritis
Oct 2000	Phase-I for Psoriasis in the US (Unknown route)
Oct 2000	Phase-I for Dermatomyositis in the US (Unknown route)
Oct 2000	Phase-I for Bullous pemphigoid in the US (Unknown route)
Oct 2000	Eculizumab has received Orphan Drug Status for Dermatomyositis in the US
Feb 2001	Preclinical development for Immune thrombocytopenic purpura in the US (Unknown route)
Mar 2001	Eculizumab has received orphan drug status for idiopathic membranous glomerular nephropathy in the US
Jan 2002	A phase IIb trial of h5G1.1 has commenced in rheumatoid arthritis
Jan 2002	Alexion has completed a pilot phase I trial in Dermatomyositis in the US
Jan 2002	Phase-II/III in Rheumatoid arthritis in the US (unspecified route)
Jan 2002	Phase-II/III in Rheumatoid arthritis in Canada (unspecified route)
Mar 2002	Enrolment has been completed in a phase II trial for membranous nephritis
Jan 2003	Alexion Pharmaceuticals has entered into a large-scale product supply agreement with Lonza Biologics
Jan 2003	Alexion has completed enrolment in a phase IIb trial for rheumatoid arthritis in the US and Canada
Jul 2003	Suspended – Phase-I for Dermatomyositis in the US (unspecified route)
Aug 2003	Eculizumab has received orphan drug status for paroxysmal nocturnal haemoglobinuria in the US
Dec 2003	Eculizumab has received orphan drug status for Paroxysmal nocturnal haemoglobinuria in the EU

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Table II. Contd

Jul 2004	The US FDA has approved two protocols, under a Special Protocol Assessment (SPA), for the pivotal phase III programme of eculizumab in Paroxysmal Nocturnal Haemoglobinuria
Nov 2004	Alexion has initiated enrolment in the TRIUMPH trial for paroxysmal nocturnal haemoglobinuria in Australia, Canada, Europe and the US
Nov 2004	Phase-III in Paroxysmal nocturnal haemoglobinuria in Canada (IV-infusion)
Nov 2004	Phase-III in Paroxysmal nocturnal haemoglobinuria in European Union (IV-infusion)
Nov 2004	Phase-III in Paroxysmal nocturnal haemoglobinuria in the US (IV-infusion)
Nov 2004	Phase-III in Paroxysmal nocturnal haemoglobinuria in Australia (IV-infusion)
Jan 2005	Alexion has initiated enrolment in the phase III SHEPHERD trial for paroxysmal nocturnal haemoglobinuria in Australia, Canada, Europe, and the US
Apr 2005	Alexion Pharmaceuticals has completed enrolment in the TRIUMPH trial for Paroxysmal Nocturnal Haemoglobinuria in the US, Canada, Europe and Australia
Jul 2005	Alexion has completed randomisation in the phase III TRIUMPH trial for paroxysmal nocturnal haemoglobinuria in Australia, Canada, Europe and the US
Sep 2005	Alexion Pharmaceuticals has completed enrolment in the SHEPHERD trial for Paroxysmal Nocturnal Haemoglobinuria in the US, Canada, Europe and Australia
Sep 2005	Discontinued – Phase-II for Lupus nephritis in the US (unspecified route)
Sep 2005	No development reported - Preclinical for Immune thrombocytopenic purpura in the US (unspecified route)
Sep 2005	Discontinued – Phase-II for Systemic lupus erythematosus in the US (unspecified route)
Sep 2005	Discontinued – Phase-II/III for Rheumatoid arthritis in the US (IV-infusion)
Sep 2005	Discontinued – Phase-I for Psoriasis in the US (unspecified route)
Sep 2005	Discontinued – Phase-I for Dermatomyositis in the US (unspecified route)
Sep 2005	Discontinued – Phase-I for Bullous pemphigoid in the US (unspecified route)
Sep 2005	Discontinued – Phase-II/III for Rheumatoid arthritis in Canada (IV-infusion)
Sep 2005	Discontinued – Phase-II for Glomerulonephritis in the US (unspecified route)
Mar 2006	This compound is still in active development for transplant rejection
Mar 2006	Prein Asthma in the US (unspecified route)
Aug 2006	Eculizumab has received accelerated assessment status for paroxysmal nocturnal haemoglobinuria in Europe
Sep 2006	Preregistration for Paroxysmal nocturnal haemoglobinuria in the US (IV-infusion)
Sep 2006	Preregistration for Paroxysmal nocturnal haemoglobinuria in European Union (IV-infusion)
Nov 2006	Alexion has been granted a priority review for eculizumab by the FDA
Nov 2006	Discontinued – Preclinical for Adult respiratory distress syndrome in the US (unspecified route)
Nov 2006	Prein Transplant rejection in the US (unspecified route)
Nov 2006	Discontinued – Preclinical for Immune thrombocytopenic purpura in the US (unspecified route)

In C5-deficient mice reconstituted with human C5, eculizumab potently inhibited the complement system when administered IV or SC at equivalent doses.[18]

1.2.2 Immunological Disorders

In C5-deficient mice reconstituted with human C5, eculizumab potently inhibited the complement system when administered IV or SC at equivalent doses.[18]

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1.2.3 Respiratory Tract Disorders

In C5-deficient mice reconstituted with human C5, eculizumab potently inhibited the complement system when administered IV or SC at equivalent doses.[18]

1.2.4 Rheumatic Disease

In C5-deficient mice reconstituted with human C5, eculizumab potently inhibited the complement system when administered IV or SC at equivalent doses.[18]

1.2.5 Transplant Rejection

In C5-deficient mice reconstituted with human C5, eculizumab potently inhibited the complement system when administered IV or SC at equivalent doses.[18] Eculizumab blocked human complement-mediated haemolytic activity and C5a generation *in vitro*.

In an *ex vivo* model of pig-to-human xenotransplantation, the addition of eculizumab (50–200 μg/mL) to human blood prior to perfusion dose-dependently prolonged graft survival time compared with no treatment. No signs of hyperacute rejection were observed in the two hearts that were perfused with blood containing the highest dose of antibody. Eculizumab reduced total complement activity, and the two higher doses reduced fluid phase C5b-9 levels compared with no treatment, indicating that the prevention of C5b-9 production correlates with increased heart survival.[19]

1.3 Therapeutic Trials

1.3.1 Genitourinary Disorders

In the double-blind, C99-004 trial, 117 patients with membranous nephritis were randomised to receive placebo, eculizumab 8 mg/kg every 4 weeks or eculizumab 8 mg/kg every 2 weeks for 4 months. The primary efficacy endpoint, 24-hour urinary protein, was not significantly different between study groups. However, subset analyses showed that patients who appeared to benefit most clinically also had more complete complement inhibition; an important finding as to the pathology of the disease and to the effectiveness of eculizumab treatment.[16]

In the E99-004 trial, 72 placebo and eculizumab patients with membranous nephritis from trial C99-004 were entered into a pre-planned, open-label extension study designed to provide eculizumab 8 mg/kg every 2 weeks for 12 months. The trial is still ongoing and after 9 months of therapy, eculizumab administration was associated with significant improvements in proteinuria, urinary protein/creatinine ratio and serum albumin. At the end of 12 months of therapy, urinary protein decreased 42% ($p < 0.001$), urinary protein/creatinine ratio decreased 45% ($p < 0.001$), serum cholesterol decreased 10% ($p = 0.005$), serum triglycerides decreased 16% ($p = 0.008$) and serum albumin decreased 14% ($p < 0.001$) in the 39 patients for whom 1 year of follow-up data was available. Remission rate was examined prospectively using the widely employed clinical definition of remission in patients with nephrotic membranous nephritis that requires both a 50% reduction in proteinuria and a 24-hour proteinuria <3.5g. For the 55 evaluable E99-004 patients who were treated with eculizumab for 6 months or longer, eculizumab treatment was associated with a 27% 12-month remission rate by Kaplan-Meier analysis.[16]

1.3.2 Haematological Disorders

Cumulative 1-year results from an ongoing open-label, phase Ib trial showed that eculizumab produced a rapid clinical response within a week of administration that lasted over the course of at least 1 year. Eculizumab significantly reduced the need for blood transfusions, intravascular haemolysis and haemoglobinuria among 11 patients with PNH. This was accompanied by an associated improvement in the quality of life, which was maintained for at least 1 year of continuous treatment. All patients received four once-weekly intravenous (IV) infusions of eculizumab 600mg, followed by a 900mg dose 1 week later and then by 900mg every other week through week 12. Levels of lactate dehydrogenase were significantly decreased from a mean of 3111 IU/L at baseline to 594 IU/L during treatment. The percentage of PNH type III erythrocytes increased significantly from a mean of 36.7% at baseline to 59.2% at the end of 12 weeks of treatment. In addition, the

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mean and median transfusion rates were reduced from baseline values of 2.1 and 1.8 units per month, respectively, to 0.6 and 0.0 units per month, respectively, during eculizumab therapy. In nine patients for whom urine scores were assessed, the mean paroxysm rate (defined as the mean number of days in paroxysm per patient per month) was significantly reduced from 2.9 days to 0.12 day. During treatment, there were significant improvements in measurements of the quality of life.[6,11,12,20] Two-year cumulative results from this study showed that eculizumab was associated with statistically significant reductions in haemolysis and in the need for blood transfusions. Two-year results showed the percentage of PNH type III erythrocytes to have increased significantly to 58.9%. The mean and median transfusion rates were reduced to 0.4 and 0.3 units/patient/month, respectively, through the 23- to 26-month treatment period.[7]

Eculizumab achieved all pre-specified primary and secondary endpoints in the TRIUMPH trial with statistical significance. The median transfusion rate for placebo was 10 units/patient compared with 0 units/patient for eculizumab. No patients in the placebo group achieved haemoglobin stabilisation, compared with 49% of patients in the eculizumab group. The TRIUMPH trial enrolled 87 patients with paroxysmal nocturnal haemoglobinuria.[3]

All pre-specified primary and secondary efficacy endpoints were achieved with statistical significance in the SHEPHERD trial at 6 months; lactate dehydrogenase was reduced by 87%.[5]

1.3.3 Immunological Disorders

A single dose of eculizumab up to 8 mg/kg was well tolerated by 24 lupus patients in a phase I double-blind, placebo-controlled ascending dose trial. A single 8 mg/kg dose of eculizumab was associated with a significant reduction in the incidence of proteinurea, an objective measurement of kidney dysfunction. A single dose of eculizumab potently blocked complement activity in patients for up to 2 weeks.[21]

1.3.4 Rheumatic Disease

In a multicentre, double-blind, placebo-controlled, ascending-dose trial, 40 patients with rheumatoid arthritis received a single dose of the antibody eculizumab (0.1–8.0 mg/kg). Eculizumab dose-dependently blocked complement activity for ≤2 weeks. The highest dose (8.0 mg/kg) significantly reduced C-reactive protein levels (the most objective component of the American College of Rheumatology's disease parameters), whereas in placebo recipients, C-reactive protein increased 24%. Eculizumab also reduced some of the more subjective measures of disease activity, such as tender joint count, swollen joint count, patient's global assessment of disease and patient's global assessment of pain.[13]

In a multicentre study, 209 patients with mild-to-moderate rheumatoid arthritis were randomised to receive IV eculizumab 8 mg/kg once weekly for 4 weeks then once monthly (induction/monthly group), 8 mg/kg once weekly for 4 weeks then once every 2 weeks (induction/biweekly group) or 8 mg/kg once every 2 weeks (biweekly group), or placebo. After 3 months' treatment, a higher proportion of patients in the induction/monthly, compared with the placebo, group met American College of Rheumatology 20% improvement criteria (ACR 20) [43% vs 20%]. Compared with the placebo group, patients in the induction/monthly and induction/biweekly groups had greater reductions from baseline in C-reactive protein levels.[22]

In a double-blind, randomised, placebo-controlled phase IIb trial in 368 patients with chronic rheumatoid arthritis undergoing treatment with methotrexate or leflunomide, eculizumab was administered either once per month or biweekly for 6 months. Monthly administration caused a significant, moderate improvement in ACR20 (34%), compared with placebo (22%), at 6 months. In addition, erythrocyte sedimentation rate (ESR) was significantly reduced. Biweekly administration of eculizumab did not significantly change ACR20 compared with placebo.[17,23]

1.3.5 Skin Disorders

Psoriasis: Treatment for 2 months with eculizumab did not significantly improve Psoriasis Area and Severity Index (PASI) scores in a study in 40 patients with severe psoriasis. However, trends

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towards improvement were observed in certain measures of disease activity. Haemolytic activity and deposition of activated terminal complement in psoriatic plaques were reduced in a dose-dependent manner.[14]

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The European Agency for the Evaluation of Medicinal Products
Evaluation of Medicines for Human Use

London, 20 February 2003
CPMP/QWP/419/03

COMMITTEE FOR PROPRIETARY MEDICINAL PRODUCTS (CPMP)

<DRAFT>

NOTE FOR GUIDANCE ON EXCIPIENTS, ANTIOXIDANTS AND ANTIMICROBIAL PRESERVATIVES IN THE DOSSIER FOR APPLICATION FOR MARKETING AUTHORISATION OF A MEDICINAL PRODUCT

DISCUSSION IN THE QUALITY WORKING PARTY	April 2002, January 2003
TRANSMISSION TO CPMP	February 2003
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Note:
This Note for Guidance replaces 3AQ9a Excipients in the Dossier for Application for Marketing Authorisation of a Medicinal Products and CPMP/CVMP/QWP/115/95 Note for Guidance on Inclusion of Antioxidants and Antimicrobial Preservatives in Medicinal Products

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NOTE FOR GUIDANCE ON EXCIPIENTS, ANTIOXIDANTS AND ANTIMICROBIAL PRESERVATIVES IN THE DOSSIER FOR APPLICATION FOR MARKETING AUTHORISATION OF A MEDICINAL PRODUCT

INTRODUCTION

This note for guidance is concerned with the application to excipients, antioxidants and antimicrobial preservatives of Module 3, sections P.1, P.2, P.4, P.5, P.8 of the Common Technical Document with a view to granting a marketing authorisation for a new medicinal product.

The data should be presented according to the standard format described in the CTD Module 3, part P.

Antioxidants and Antimicrobial Preservatives are substances which are used to extend the shelf-life of medicines by respectively retarding the oxidation of active substances and excipients, and by reducing microbial proliferation.

The properties of these substances are due to certain chemical groups which are usually aggressive towards living cells and which lead to certain risks when used in man.

If it is not absolutely necessary to add these substances in medicinal products they must be avoided.

The purpose of this note for guidance is to describe the information that needs to appear in application for marketing authorisations with regards to the addition of any antioxidants or antimicrobial preservatives.

For each antioxidants and antimicrobial preservative the application should contain :

- reason for inclusion
- proof of efficacy
- the method of control in finished product
- details of the labelling of the finished product
- safety information

Several guidelines should be also taken into account :

- ICH Topic Q 6 A : Specifications : Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products : Chemical Substances (3.3.2.2. Oral liquids : d) Antimicrobial preservative content, e) Antioxidants preservative content ; 3.3.2.3. Parenteral Drug Products : g) h) and Decision Tree 8 : Microbiological Attributes of Non-sterile drug products).
- Note for Guidance on Development Pharmaceutics : 3.3. Liquid and Semi-solid Formulations.
- 3BC7A : Excipients in the Label and Package leaflet of Medicinal Products for Human Use
- Note for guidance on maximum shelf-life for sterile products after first opening or following reconstitution.

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SECTION P.1 Description and Composition of the Drug Product

Excipients must be listed, specifying their common name, their quantity and the use and reference to any relevant standard. When the common name is not sufficient to indicate functional specifications, the brand name with commercial grade should be specified. In the case of excipients presented as a mixture of compounds, details as to the composition should be provided in qualitative and quantitative terms. However, for flavouring agents and aromatic substances, it is permitted to give the qualitative composition only.

Antimicrobial preservatives and antioxidants should be chemically defined (reference to existing European Pharmacopoeia monographs may be used) and designated by the Chemical Abstract Service Registry Number (RN-CAS).

The purpose for the inclusion of any antioxidant or microbial preservative should be stated (antioxidant for the benefit of active substance or excipient or both, or antimicrobial preservative).

SECTION P.2 Pharmaceutical development

SECTION P.2.1 Components of the Drug Product

The section P.2.1.2 should comprise an explanation of the choice of the excipient (and grade where necessary) and the level of excipient according to the note for guidance "Development pharmaceutics and process validation".

For antimicrobial preservatives and antioxidants, during the pharmaceutical development of the product the applicant should demonstrate:

- the necessity to add an antioxidant or preservative to the finished product at the chosen level
- the physical and chemical compatibility of the antioxidant and of the preservative with other constituents of the finished product, the container and the closures.

The used concentration must be justified in terms of efficacy and safety, such that the minimum concentration of preservative is used which gives the required level of efficacy. The appropriate test method for efficacy of antimicrobial preservation is that of the European Pharmacopoeia. This should be used to determine whether the required level of activity is achieved.

In the case of antioxidants, these should only be used once it has been shown that their use cannot be avoided, even if the manufacturing process is optimised to minimise the potential for oxidation, for instance by manufacturing and filling products under an inert headspace gas.

The safety of the antioxidant or preservative should be supported by bibliographic and /or experimental data.

Some antioxidants or antimicrobial preservatives may be undesirable under certain circumstances :

- mercury containing preservatives : see the CPMP Position Paper on Thiomersal, Implementation of the Warning Statement Relating to Sensitisation (CPMP/2612/99) and EMEA Position Statement on recent developments concerning thiomersal in vaccines (EMEA/CPMP/1578/00). This kind of preservative should be strictly avoided except if no other possibility may be considered but in this case the choice should be fully justified.

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- benzyl alcohol : when used in parenteral products for children under the age of two years. Its degradation product and metabolite is the benzaldehyde, toxic for the CNS.
- Benzoic acid esters (Para hydroxy benzoate and their derivates etc) : when used in any dosage-form for parenteral use.
- Sulphites and Metabisulphites.

Parenteral infusions do not contain any added antimicrobial preservatives and no antimicrobial preservatives are added when the medicinal product is intended for administration by routes where for medical reasons an antimicrobial preservative is unacceptable, such as intercisternally or by any other route of administration which gives access to the cerebrospinal fluid or retro-ocularly.

SECTION P.4 Control of Excipients

Examples of different kinds of excipients are given in the annex.

1. Specifications (P.4.1), Analytical Procedures (P.4.2), Validation of Analytical Procedures (P.4.3) and Justification of Specifications (P.4.4) :

1.1 Excipients described in the European Pharmacopoeia or, if not described in the European Pharmacopoeia, pharmacopoeia of a Member State

The routine tests which are to be carried out on each batch of starting materials must be stated in the application for marketing authorisation. If tests other than those mentioned in the pharmacopoeia are used, proof must be supplied that the test methods used are suitable to establish that the starting materials meet the quality requirements of that pharmacopoeia. When the monograph covers a family of related products, the particular specifications chosen for the excipients must be submitted. In addition and when necessary, the test used to determine the quality of the excipient should be shown to be in relation to the function that it fulfils in the medicinal product.

Data on microbiological contamination of the excipients used in the manufacture of sterile products should always be given where membrane filtration is used to achieve sterility.

Antimicrobial preservatives and antioxidants are defined as excipients and as such should be controlled following the rules governing medicinal products in the European Union. These data should be provided in part P.4.

1.2 Excipients not described in the European Pharmacopoeia or in the pharmacopoeia of a Member State

An appropriate specification of the excipient must be established, based on the following types of tests:

* Physical characteristics
* Identification tests
* Purity tests, including limits for total or individual impurities, which should be named. Purity tests may be physical, chemical, biological and, if appropriate, immunological.

  Where sterile filtration is used in the manufacture of a parental medicinal product, data and routine tests on microbiological contamination of excipients should always be given.
* Other relevant tests including, e.g. the tests on parameters which may influence the performance of the dosage form.

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* Assay or limit tests if necessary.

When an excipient is not described in the European Pharmacopoeia or in the pharmacopoeia of a Member State or in an other compendium of established use (e.g. USNF Pharmacopoeia and Japanese Pharmacopoeia), validation data of the test methods used should be presented, where appropriate.

1.3 Justification of Specifications

Justifications of specifications takes into account the choice and use of an excipient which is used for a particular purpose: it will determine the properties which must be checked during the routine tests and which will be the subject of certain specifications in connection with the bioavailability of the product (see Note for Guidance "Specifications and control tests on the finished product").

Nevertheless, justification of specifications are not systematically required for well-known excipients. For example, they are not required for excipients which have been used in similar medicinal products for a long period of time and when their characteristics and properties have not changed significantly.

For solid and semi-solid dosage forms, the justification of specifications should, if necessary, provide information on the relevant characteristics of the excipient. Special tests are often necessary (e.g. to verify the capacity of the excipient to emulsify and disperse, or to measure the viscosity...).

Appropriate data are needed for excipients used in a new route of administration.

Justification of specifications on excipients already included in the European Pharmacopoeia, or if not included in the European Pharmacopoeia, in the pharmacopoeia of a Member State and other well-known excipients already used in a medicinal product.

For these excipients, justification of specifications will normally not be required. However, any particular specification concerning the characteristics, as defined in Section P.2.1.2, should be justified (e.g. sieve analysis, in relation to microcrystallinity).

Excipients of Human or Animal Origin (P.4.5)

Viral Safety and TSE Risk should be documented in accordance with the relevant directives.

Novel Excipients (P.4.6)

For novel excipients : a dossier should be established containing the same data as required for new active substances:

a) A strict definition of the excipient, its function and its conditions of use. If the excipient is complex or is made of a mixture of compounds, the composition must be specified in qualitative and quantitative terms.

b) For new excipients and for excipients presented as a mixture of compounds the following should be taken into consideration:

   i. Any bibliographical data on the chemistry and on the toxicology and the field in which the product is already used.

   ii. The Community provisions concerning additives in foodstuff: any criteria which are based on the toxicological data, with cross-references to these data.

   The quality specifications which have been laid down in the directives are satisfactory as long as the routine control tests used are validated.

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iii. The international specifications (FAO/WHO/JECFA), and other publications such as the Food Chemical Codex.

iv. For medicinal products for topical use, data on the starting material in cosmetic products (Directive 76/768/EEC).

v. Data concerning the toxicology of the new excipient should be presented according to the dosage form and the route of administration of the medicinal product (if applicable).

c) Documentation on chemistry of excipients is required for all new excipients, taking as its basis the Note for Guidance "Chemistry of Active Substances".

* The origin of the excipient, including the name and address of manufacturer.
* A general outline of the synthesis (manufacture and purification).
* Structure.
* Physical, chemical properties, identification and purity tests.
* Validated methods of analysis with a presentation of batch results.
* Miscellaneous information (microbiological tests, etc).
* Contamination, presence of foreign substances, residual solvents, etc.
* In the case of an excipient obtained from a mixture of several components, the quality of each component and the physico-chemical tests for the mixture should be described.

The routine test procedures and limits should be established on the basis of the documentation given in the dossier.

SECTION P.5 Control of Drug Product

Apart from those situations envisaged in the note for guidance "Specifications and control tests on the finished product" it is not usually necessary to carry out identity testing and an assay of the excipients in the finished product at release.

Nevertheless, the finished product release specifications should include an identification test and limits for any antioxidants and antimicrobial preservatives present in the formulation. The finished product specification against which the product is tested throughout its shelf-life should also include limits for the antimicrobial preservatives present.

Where antioxidants are used up during the manufacture of the product, the release limits should be justified by batch data. The adequacy of specified limits should be justified on the basis of controlled conditions and in-use stability testing to ensure that sufficient antioxidant remains to protect the product throughout its entire shelf-life and during the proposed in-use period.

The control of antioxidants and antimicrobial preservatives should comply with the requirements identified in the guideline : ICH Topic Q 6 A : Specifications : Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products : Chemical Substances .

The antimicrobial preservative and antioxidants must be routinely identified and quantified at release.

P.8 Stability

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The maintenance of the physico-chemical properties of the finished product depend on the properties and the stability of the excipients (see note for guidance "Specifications and control tests on the finished product").

For new excipients, stability data should be provided as required for new active substances.

The application should follow the current QWP/CPMP guidelines on the stability of new dosage forms and should ensure that antimicrobial preservative and antioxidants levels are quantified periodically throughout the shelf-life of the finished product. In addition the efficacy of the preservatives should be established using the European Pharmacopoeia test for efficacy of antimicrobial preservation. This should be performed on the finished product at the end of the shelf-life and at the lower preservative limit in the end of shelf-life specification. The former is necessary, even if no evidence of degradation of the antimicrobial preservative and of the antioxidant is observed on storage, as other chemical and physical changes in the finished product may influence the efficacy of the antimicrobial preservative and the antioxidant.

In the case of products presented in multidose containers, the efficacy of the antimicrobial preservative under simulated in-use conditions must be established. The tests should be performed under the same condition as it is expected to be used by the final user. It may also be appropriate to examine the efficacy of the antimicrobial preservative following storage of opened or used containers for the proposed in use shelf-life.

R LABELLING

- Labelling must in accordance with relevant EC directive 2001/83/EC, Note for Guidance : Excipients in the Label and Package leaflet of Medicinal Products for Human Use and CPMP Position Paper on Thiomersal, Implementation of the Warning Statement Relating to Sensitisation (CPMP/2612/99).

However, if a product is presented in a multidose container without a preservative because :

a) it is intended for single use only (e.g. cytotoxic),

b) the product is self-preserving,

c) the product is oils based,

the labelling and product literature should indicate the absence of a preservative. This would not only emphasise the increased risk associated with the use of such products, but also aid the physician to specifically identify a product without preservative.

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ANNEX

EXAMPLES OF REQUIREMENTS CONCERNING DIFFERENT KINDS OF EXCIPIENTS

1. Excipients, which are a single chemical entity, include, for example, organic and inorganic acids and their salts, sugars and alcohols.

   They may have undergone physical treatments, which gave them special technological characteristics (e.g. micronisation).

2. Chemically transformed excipients include excipients which have undergone a special chemical treatment in order to confer certain technological characteristics (e.g. modified starch). The name and quality of such excipients should be defined in such a way as to avoid confusion with an unmodified excipient.

3. Mixtures of chemically related components include, for example, polyol esters (mixture of mono, di and tri esters), hydrogenated glucose syrup, maltitolsyrup. For these products the dossier should specify the following characteristics of the excipient:

   * the nature and content of each component with a statement of its acceptable limits;
   * technological criteria (appropriate criteria to the performance of dosage form);
   * any additives which may be present.

4. Mixed excipients are ready-for-use preparations, for example for direct compression or film coating.

   * The qualitative and quantitative composition of the mixed excipient should be submitted, the specifications of the product as a whole and of each component must be stated.

5. Excipients of natural origin, so called "natural" products have often undergone some kind of chemical treatment. In general and if relevant for the quality control of the product, data should give an outline of the operations carried out to obtain and to purify the product, and any special characteristics: decomposition products, specific impurities, chemical substances used during the treatment with residual limits, methods of sterilisation or decontamination, with a description of the effect of these processes on the excipient (e.g. modification of the physical structure).

6. For biological excipients of animal or human origin, the risk of transmitting adventitious agents should be considered and appropriate documentation submitted (e.g.; method of preparation and control of tissues and body fluids used as starting material). In addition, the name of the manufacturer and site of manufacture should be specified.

7. Flavouring agents (flavours and aromatic substances) are either natural products and/or products obtained by chemical synthesis. Because of the complexity of their composition, it is only necessary to describe the general qualitative composition mentioning the main constituents with an appropriate process of identification to ensure the consistency of the composition (in particular, identification of the main constituents and if necessary carriers).

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Most constituents of artificial flavours have internationally accepted purity criteria in food use (FAO/WHO). Reference to these standards is acceptable for medicinal products.

8. Colouring matters: - Community legislation on colouring matters in medicinal products is applicable.

ANTIOXIDANTS

Antioxidants are used to reduce the oxidation of active substances and excipients in the finished product. Antioxidants should not be used to disguise poorly formulated products or inadequate packaging. The need to include an antioxidant should be explained and fully justified. Oxidative degradation can be accelerated by light and by the presence of mineral or metallic impurities, due to the formation of free radicals.

There are three types of antioxidants :

Type	Definition	Example
True antioxidants	These are thought to block chain reactions by reacting with free radicals	Butylated hydroxytoluene (BHT)
Reducing agents	These have a lower redox potential than the drug or excipient they are protecting	Ascorbic acid
Antioxidants synergists	These enhance the effects of antioxidants	Sodium edetate

The efficacy obtained for an antioxidants depends on its nature, the stage at which it is incorporated within the finished product, the nature of the container and the formulation.

The efficacy of antioxidants must be assessed in the finished product in conditions, which simulate actual use by measuring the extent of degradation in the finished product, with and without the antioxidant.

Antioxidants should only be included in a formulation if it has been proven hat their use cannot be avoided. This applies to cases where the manufacturing process is optimised to minimise the potential for oxidation.

ANTIMICROBIAL PRESERVATIVES

Antimicrobial Preservatives are used to prevent or inhibit the growth of micro-organisms which could present a risk of infection or degradation of the medicinal product. These micro-organisms may proliferate during normal conditions of use of the product by the patient, particularly in multidose preparations.

On no account should preservatives be used as an alternative to Good Manufacturing Practice (GMP).

Preparations at greatest risk of contamination are those which contain water such as solutions, suspensions and emulsions to be taken orally, solution for external use, creams and sterile preparations used repeatedly (e.g. injectable multidose preparations and eye-drops).

The level of efficacy will vary according to the chemical structure of the preservative, its concentration, the physical and chemical characteristics of the medicinal product (especially pH) and the type and level of initial microbial contamination. The design of the pack and the

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temperature at which the product is stored will also affect of activity of any antimicrobial preservatives present.

The antimicrobial efficacy of the preservative in the finished should be assessed during product development, particularly during stability studies and at the end of the proposed shelf-life, using Pharmacopoeia European test.

If products do not contain a preservative and do not have inherent preservative efficacy they must not be packaged in multidose presentations without a sound justification.

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HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use SOLIRIS safely and effectively. See full prescribing information for SOLIRIS.

Soliris® (eculizumab) injection, for intravenous use

Initial U.S. Approval: 2007

WARNING: SERIOUS MENINGOCOCCAL INFECTIONS

See full prescribing information for complete boxed warning

Life-threatening and fatal meningococcal infections have occurred in patients treated with Soliris and may become rapidly life-threatening or fatal if not recognized and treated early (5.1).

- **Comply with the most current Advisory Committee on Immunization Practices (ACIP) recommendations for meningococcal vaccination in patients with complement deficiencies (5.1).**
- **Immunize patients with meningococcal vaccines at least 2 weeks prior to administering the first dose of Soliris, unless the risks of delaying Soliris therapy outweigh the risk of developing a meningococcal infection. [see *Warnings and Precautions (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).

INDICATIONS AND USAGE

Soliris is a complement inhibitor indicated for:

- The treatment of patients with paroxysmal nocturnal hemoglobinuria (PNH) to reduce hemolysis (1.1).
- The treatment of patients with atypical hemolytic uremic syndrome (aHUS) to inhibit complement-mediated thrombotic microangiopathy (1.2).

Limitation of Use

Soliris is not indicated for the treatment of patients with Shiga toxin *E. coli* related hemolytic uremic syndrome (STEC-HUS).

DOSAGE AND ADMINISTRATION

Only administer as an intravenous infusion

PNH Dosage Regimen: (2.1)
aHUS Dosage Regimen: (2.2)

D24

DOSAGE FORMS AND STRENGTHS

300 mg single-dose 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 (≥20%) are: headache, diarrhea, hypertension, upper respiratory infection, abdominal pain, vomiting, nasopharyngitis, anemia, cough, peripheral edema, nausea, urinary tract infections, pyrexia (6.1).

To report SUSPECTED ADVERSE REACTIONS, contact Alexion Pharmaceuticals, Inc. at) 1-844-259-6783 or FDA at 1-800-FDA-1088 or www.fda.gov/medwatch.

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).

See 17 PATIENT COUNSELING INFORMATION and Medication Guide.

Revised: 01/2017

FULL PRESCRIBING INFORMATION: CONTENTS*

WARNING: SERIOUS MENINGOCOCCAL INFECTIONS

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- 1.2 Atypical Hemolytic Uremic Syndrome (aHUS)

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FULL PRESCRIBING INFORMATION

WARNING: SERIOUS MENINGOCOCCAL INFECTIONS

Life-threatening and fatal meningococcal infections have occurred in patients treated with Soliris. Meningococcal infection may become rapidly life-threatening or fatal if not recognized and treated early [*see Warnings and Precautions* (5.1)].

- **Comply with the most current Advisory Committee on Immunization Practices (ACIP) recommendations for meningococcal vaccination in patients with complement deficiencies.**
- **Immunize patients with meningococcal vaccines least 2 weeks prior to administering the first dose of Soliris, unless the risks of delaying Soliris therapy outweigh the risk of developing a meningococcal infection. [see *Warnings and Precautions (5.1)* for additional guidance on the management of the risk of meningococcal infection].**
- **Monitor patients for early signs of meningococcal infections and evaluate immediately if infection is suspected.**

Soliris is available only through a restricted program under a Risk Evaluation and Mitigation Strategy (REMS). Under the Soliris REMS, prescribers must enroll in the program [*see Warnings and Precautions (5.2)*]. Enrollment in the Soliris REMS program and additional information are available by telephone: 1-888-SOLIRIS (1-888-765-4747) or at solirisrems.com.

1 INDICATIONS AND USAGE

1.1 Paroxysmal Nocturnal Hemoglobinuria (PNH)

Soliris is indicated for the treatment of patients with paroxysmal nocturnal hemoglobinuria (PNH) to reduce hemolysis.

1.2 Atypical Hemolytic Uremic Syndrome (aHUS)

Soliris is indicated for the treatment of patients with atypical hemolytic uremic syndrome (aHUS) to inhibit complement-mediated thrombotic microangiopathy.

Limitation of Use

Soliris is not indicated for the treatment of patients with Shiga toxin *E. coli* related hemolytic uremic syndrome (STEC-HUS).

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.

Administer Soliris 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

Administer Soliris at the recommended dosage regimen time points, or within two days of these time points.

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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 infusion of fresh frozen plasma	60 minutes prior to each infusion of fresh frozen plasma

2.3 Preparation and Administration

Dilute Soliris to a final admixture concentration of 5 mg/mL using the following steps:

- Withdraw the required amount of Soliris from the vial into a sterile syringe.
- Transfer the recommended dose to an infusion bag.
- Dilute Soliris to a final concentration of 5 mg/mL by adding the appropriate amount (equal volume of diluent to drug volume) of 0.9% Sodium Chloride Injection, USP; 0.45% Sodium Chloride Injection, USP; 5% Dextrose in Water Injection, USP; or Ringer's Injection, USP to the infusion bag.

The final admixed Soliris 5 mg/mL infusion volume is 60 mL for 300 mg doses, 120 mL for 600 mg doses, 180 mL for 900 mg doses or 240 mL for 1200 mg doses (Table 3).

Table 3: Preparation and Reconstitution of Soliris

Soliris Dose	Diluent Volume	Final Volume
300 mg	30 mL	60 mL
600 mg	60 mL	120 mL
900 mg	90 mL	180 mL
1200 mg	120 mL	240 mL

Gently invert the infusion bag containing the diluted Soliris solution to ensure thorough mixing of the product and diluent. Discard any unused portion left in a vial, as the product contains no preservatives.

Prior to administration, the admixture should be allowed to adjust to room temperature [18°-25° C, 64-77° F]. The admixture must not be heated in a microwave or with any heat source other than ambient air temperature.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

2.4 Administration

Do Not Administer As An Intravenous Push or Bolus Injection

Administer the Soliris admixture by intravenous infusion over 35 minutes in adults and 1 to 4 hours in pediatric patients via gravity feed, a syringe-type pump, or an infusion 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 in adults. Monitor the patient for at least one hour following completion of the infusion for signs or symptoms of an infusion reaction.

3 DOSAGE FORMS AND STRENGTHS

Soliris is supplied as 300 mg single-dose 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

Life-threatening and fatal meningococcal infections have occurred in patients treated with Soliris. The use of Soliris increases a patient's susceptibility to serious meningococcal infections (septicemia and/or meningitis).

Vaccinate for meningococcal disease according to the most current Advisory Committee on Immunization Practices (ACIP) recommendations for patients with complement deficiencies. Revaccinate patients in accordance with ACIP recommendations, considering the duration of Soliris therapy.

Immunize patients without a history of meningococcal vaccination at least 2 weeks prior to receiving the first dose of Soliris. If urgent Soliris therapy is indicated in an unvaccinated patient, administer meningococcal vaccine(s) as soon as possible.

In prospective clinical studies, 75/100 patients with aHUS were treated with Soliris less than 2 weeks after meningococcal vaccination and 64 of these 75 patients received antibiotics for prophylaxis of meningococcal infection until at least 2 weeks after meningococcal vaccination. The benefits and risks of antibiotic prophylaxis for prevention of meningococcal infections in patients receiving Soliris have not been established.

Vaccination reduces, but does not eliminate, the risk of meningococcal infections. In clinical studies, 2 out of 196 PNH patients developed serious meningococcal infections while receiving treatment with Soliris; both had been vaccinated [*see Adverse Reactions (6.1)*]. In clinical studies among non-PNH patients, meningococcal meningitis occurred in one unvaccinated patient. In addition, 3 out of 130 previously vaccinated patients with aHUS developed meningococcal infections while receiving treatment with Soliris [*see Adverse Reactions (6.1)*].

Closely monitor patients for early signs and symptoms of meningococcal infection and evaluate patients immediately if an infection is suspected. Meningococcal infection may become rapidly life-threatening or fatal if not recognized and treated early. Discontinue Soliris in patients who are undergoing treatment for serious meningococcal infections.

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) or at solirisrems.com.

5.3 Other Infections

Soliris blocks terminal complement activation; therefore patients may have increased susceptibility to infections, especially with encapsulated bacteria. Additionally, Aspergillus infections have occurred in immunocompromised and neutropenic patients. Children treated with Soliris may be at increased risk of developing serious infections due to *Streptococcus pneumoniae* and *Haemophilus 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 [*see Warnings and Precautions (5.1)*].

5.4 Monitoring Disease Manifestations 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 trials, 18 patients (5 in the prospective studies) discontinued Soliris treatment. TMA complications occurred following a missed dose in 5 patients, and Soliris was reinitiated in 4 of these 5 patients.

Clinical signs and symptoms of TMA include changes in mental status, seizures, angina, dyspnea, or thrombosis. In addition, the following changes in laboratory parameters may identify a TMA complication: occurrence of two, or repeated measurement of any one of the following: a decrease in platelet count by 25% or more compared to baseline or the peak platelet count during Soliris treatment; an increase in serum creatinine by 25% or more compared to baseline or nadir during Soliris treatment; or, an increase in serum LDH by 25% or more over baseline or nadir during Soliris treatment.

If TMA complications occur after Soliris discontinuation, consider reinstitution of Soliris treatment, plasma therapy [plasmapheresis, plasma exchange, or fresh frozen plasma infusion (PE/PI)], or appropriate organ-specific supportive measures.

5.5 Thrombosis Prevention and Management

The effect of withdrawal of anticoagulant therapy during Soliris treatment has not been established. Therefore, treatment with Soliris should not alter anticoagulant management.

5.6 Infusion Reactions

As with all protein products, administration of Soliris may result in infusion reactions, including anaphylaxis or other hypersensitivity reactions. In clinical trials, no patients experienced an infusion reaction which required discontinuation of Soliris. Interrupt Soliris infusion and institute appropriate supportive measures if signs of cardiovascular instability or respiratory compromise occur.

6 ADVERSE REACTIONS

The following serious adverse reactions are discussed in greater detail in other sections of the labeling:

- Serious Meningococcal Infections [*see Warnings and Precautions (5.1)*]
- Other Infections [*see Warnings and Precautions (5.3)*]
- Monitoring Disease Manifestations After Soliris Discontinuation [*see Warnings and Precautions (5.4)*]
- Thrombosis Prevention and Management [*see Warnings and Precautions (5.5)*]
- Infusion Reactions [*see Warnings and Precautions (5.6)*]

6.1 Clinical Trial Experience

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice.

Meningococcal infections are the most important adverse reactions experienced by patients receiving Soliris. In PNH clinical studies, two patients experienced meningococcal sepsis. Both patients had previously received a meningococcal vaccine. In clinical studies among patients without PNH, meningococcal meningitis occurred in one unvaccinated patient. Meningococcal sepsis occurred in one previously vaccinated patient enrolled in the retrospective aHUS study during the post-study follow-up period [*see Warnings and Precautions (5.1)*].

PNH

The data described below reflect exposure to Soliris in 196 adult patients with PNH, age 18-85, of whom 55% were female. All had signs or symptoms of intravascular hemolysis. Soliris was studied in a placebo-controlled clinical study (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.

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 four prospective, single-arm studies, three in adult and adolescent patients (aHUS Studies 1, 2, and 4), one in pediatric and adolescent patients (aHUS Study 5) and one retrospective study (aHUS Study 3).

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The data described below were derived from 78 adult and adolescent patients with aHUS enrolled in aHUS Study 1, aHUS Study 2, and aHUS Study 4. All patients received the recommended dosage of Soliris. Median exposure was 67 weeks (range: 2-145 weeks). Table 5 summarizes all adverse events reported in at least 10% of patients in aHUS Studies 1, 2, and 4 combined.

Table 5: Per Patient Incidence of Adverse Events in 10% or More Adult and Adolescent Patients Enrolled in aHUS Study 1, aHUS Study 2 and aHUS Study 4 Separately and in Total

MedDRA ver. 15.1	Number (%) of Patients			
	Study 1 (n=17)	Study 2 (n=20)	Study 4 (n=41)	Total (n=78)
Vascular Disorders				
Hypertension[a]	10 (59)	9 (45)	7 (17)	26 (33)
Hypotension	2 (12)	4 (20)	7 (17)	13 (17)
Infections and Infestations				
Bronchitis	3 (18)	2 (10)	4 (10)	9 (12)
Nasopharyngitis	3 (18)	11 (55)	7 (17)	21 (27)
Gastroenteritis	3 (18)	4 (20)	2 (5)	9 (12)
Upper respiratory tract infection	5 (29)	8 (40)	2 (5)	15 (19)
Urinary tract infection	6 (35.3)	3 (15)	8 (20)	17 (22)
Gastrointestinal Disorders				
Diarrhea	8 (47)	8 (40)	12 (32)	29 (37)
Vomiting	8 (47)	9 (45)	6 (15)	23 (30)
Nausea	5 (29)	8 (40)	5 (12)	18 (23)
Abdominal pain	3 (18)	6 (30)	6 (15)	15 (19)
Nervous System Disorders				
Headache	7 (41)	10 (50)	15 (37)	32 (41)
Blood and Lymphatic System Disorders				
Anemia	6 (35)	7 (35)	7 (17)	20 (26)
Leukopenia	4 (24)	3 (15)	5 (12)	12 (15)
Psychiatric Disorders				
Insomnia	4 (24)	2 (10)	5 (12)	11 (14)
Renal and Urinary Disorders				
Renal impairment	5 (29)	3 (15)	6 (15)	14 (18)
Proteinuria	2 (12)	1 (5)	5 (12)	8 (10)
Respiratory, Thoracic and Mediastinal Disorders				
Cough	4 (24)	6 (30)	8 (20)	18 (23)
General Disorders and Administration Site Conditions				
Fatigue	3 (18)	4 (20)	3 (7)	10 (13)
Peripheral edema	5 (29)	4 (20)	9 (22)	18 (23)
Pyrexia	4 (24)	5 (25)	7 (17)	16 (21)
Asthenia	3 (18)	4 (20)	6 (15)	13 (17)
Eye Disorder	5 (29)	2 (10)	8 (20)	15 (19)
Metabolism and Nutrition Disorders				
Hypokalaemia	3 (18)	2 (10)	4 (10)	9 (12)
Neoplasms benign, malignant, and unspecified (including cysts and polyps)	1 (6)	6 (30)	1 (20)	8 (10)
Skin and Subcutaneous Tissue Disorders				
Rash	2 (12)	3 (15)	6 (15)	11 (14)
Pruritis	1 (6)	3 (15)	4 (10)	8 (10)
Musculoskeletal and Connective Tissue Disorders				
Arthralgia	1 (6)	2 (10)	7 (17)	10 (13)
Back pain	3 (18)	3 (15)	2 (5)	8 (10)

[a] includes the preferred terms hypertension, accelerated hypertension, and malignant hypertension.

In aHUS Studies 1, 2, and 4 combined, 60% (47/78) of patients experienced a serious adverse event (SAE). The most commonly reported SAEs were infections (24%), hypertension (5%), chronic renal failure (5%), and renal impairment (5%). Five patients discontinued Soliris due to adverse events; three due to worsening renal function, one due to new diagnosis of Systemic Lupus Erythematosus, and one due to meningoccal meningitis.

aHUS Study 5 included 22 pediatric and adolescent patients, of which 18 patients were less than 12 years of age. All patients received the recommended dosage of Soliris. Median exposure was 44 weeks (range: 1 dose-87 weeks). Table 6 summarizes all adverse events reported in at least 10% of patients enrolled in aHUS Study 5.

Table 6: Per Patient Incidence of Adverse Reactions in 10% or More Patients Enrolled in aHUS Study 5

MedDRA ver. 15.1	1 month to <12 yrs (n=18)	Total (n=22)
Eye Disorders	3 (17)	3 (14)
Gastrointestinal Disorders		
Abdominal pain	6 (33)	7 (32)
Diarrhoea	5 (28)	7 (32)
Vomiting	4 (22)	6 (27)
Dyspepsia	0	3 (14)
General Disorders and Administration Site Conditions		
Pyrexia	9 (50)	11 (50)
Infections and Infestations		
Upper respiratory tract infection	5 (28)	7 (32)
Nasopharyngitis	3 (17)	6 (27)
Rhinitis	4 (22)	4 (18)
Urinary Tract infection	3 (17)	4 (18)
Catheter site infection	3 (17)	3 (14)
Musculoskeletal and Connective Tissue Disorders		
Muscle spasms	2 (11)	3 (14)
Nervous System Disorders		
Headache	3 (17)	4 (18)
Renal and Urinary Disorders	3 (17)	4 (18)
Respiratory, Thoracic and Mediastinal Disorders		
Cough	7 (39)	8 (36)
Oropharyngeal pain	1 (6)	3 (14)
Skin and Subcutaneous Tissue Disorders		
Rash	4 (22)	4 (18)
Vascular Disorders		
Hypertension	4 (22)	4 (18)

In aHUS Study 5, 59% (13/22) of patients experienced a serious adverse event (SAE). The most commonly reported SAEs were hypertension (9%), viral gastroenteritis (9%), pyrexia (9%), and upper respiratory infection (9%). One patient discontinued Soliris due to an adverse event (severe agitation).

Analysis of retrospectively collected adverse event data from pediatric and adult patients enrolled in 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 7.

Table 7: 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 (40)	1 (25)	9 (47)
Gastrointestinal Disorders				
Diarrhea	1 (20)	4 (40)	1 (25)	6 (32)
Vomiting	2 (40)	1 (10)	1 (25)	4 (21)
Infections and Infestations				
Upper respiratory tract infection[a]	2 (40)	3 (30)	1 (25)	6 (32)
Respiratory, Thoracic and Mediastinal Disorders				
Cough	3 (60)	2 (20)	0 (0)	5 (26)
Nasal congestion	2 (40)	2 (20)	0 (0)	4 (21)
Cardiac Disorders				
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 with eculizumab. The immunogenicity of Soliris has been evaluated using two different immunoassays for the detection of anti-eculizumab antibodies: a direct enzyme-linked immunosorbent assay (ELISA) using the Fab fragment of eculizumab as target was used for the PNH indication; and an electro-chemiluminescence (ECL) bridging assay using the eculizumab whole molecule as target was used for the aHUS indication, as well as for additional patients with PNH. In the PNH population, antibodies to Soliris were detected in 3/196 (2%) patients with PNH treated with Soliris using the ELISA assay and in 5/161 (3%) patients treated with Soliris using the ECL assay. In patients with aHUS treated with Soliris, antibodies to Soliris were detected in 3/100 (3%) using 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 3 patients with aHUS and also for the 5 patients with PNH with positive samples using the ECL assay. 2/161 patients in the PNH group (1.2%) and 1/100 patients in the aHUS group (1%) had low positive values for neutralizing antibodies. No apparent correlation of antibody development to clinical response was observed in either indication. 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 and 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

The following adverse reactions have been identified during post-approval use of Soliris. Because these reactions are reported voluntarily from a population of uncertain size, it is not always possible to reliably estimate their frequency or establish a causal relationship to Soliris exposure.

Cases of serious or fatal meningococcal infections have been reported.

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Pregnancy Category C

Risk Summary

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 Data

Animal reproduction studies were conducted in mice using doses of a murine anti-C5 antibody that approximated 2-4 times (low dose) and 4-8 times (high dose) the recommended human Soliris dose, based on a body weight comparison. When animal exposure to the antibody occurred in the time period from before mating until early gestation, no decrease in fertility or reproductive performance was observed. When maternal exposure to the antibody occurred during organogenesis, two cases of retinal dysplasia and one case of umbilical hernia were observed among 230 offspring born to mothers exposed to the higher antibody dose; however, the exposure did not increase fetal loss or neonatal death. When maternal exposure to the antibody occurred in the time period from implantation through weaning, a higher number of male offspring became moribund or died (1/25 controls, 2/25 low dose group, 5/25 high dose group). Surviving offspring had normal development and reproductive function.

8.3 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

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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 have not been established.

The safety and effectiveness of Soliris for the treatment of aHUS have been established in pediatric patients. Use of Soliris in pediatric patients for this indication is supported by evidence from four adequate and well-controlled clinical studies assessing the safety and effectiveness of Soliris for the treatment of aHUS. The studies included a total of 47 pediatric patients (ages 2 months to 17 years). The safety and effectiveness of Soliris for the treatment of aHUS appear similar in pediatric and adult patients [*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

Nineteen patients 65 years of age or older (15 with PNH and 4 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-dose 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 Dosage and Administration (2.2)*].

Dedicated studies have not been conducted to evaluate the PK of Soliris in special patient populations identified by gender, race, age (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 Paroxysmal Nocturnal Hemoglobinuria (PNH)

The safety and efficacy of Soliris in PNH patients with hemolysis were assessed in a randomized, double-blind, placebo-controlled 26 week study (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 8).

Table 8: 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 9). 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 9: PNH Study 1 Results

	Placebo N = 44	Soliris N = 43
Percentage of patients with stabilized hemoglobin levels	0	49
Packed RBC units transfused per patient (median)	10	0
(range)	(2 - 21)	(0 - 16)
Transfusion avoidance (%)	0	51
LDH levels at end of study (median, U/L)	2,167	239
Free hemoglobin at end of study (median, mg/dL)	62	5

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 Atypical Hemolytic Uremic Syndrome (aHUS)

Five single-arm studies [four prospective (aHUS Studies 1, 2, 4 and 5) 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 and Study 5 was based on body weight [*see Dosage and Administration (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 were required to have ADAMTS13 activity level above 5%; observed range of values in the trial were 70%-121%. Seventy-six percent of patients had an identified complement regulatory factor mutation or auto-antibody. Table 10 summarizes the key baseline clinical and disease-related characteristics of patients enrolled in aHUS Study 1.

Table 10: 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. In aHUS Study 1, the median duration of Soliris therapy was approximately 100 weeks (range: 2 weeks to 145 weeks).

Renal function, as measured by eGFR, was improved and maintained during Soliris therapy. The mean eGFR (± SD) increased from 23 ± 15 mL/min/1.73m² at baseline to 56 ± 40 mL/min/1.73m² by 26 weeks; this effect was maintained through 2 years (56 ± 30 mL/min/1.73m²). Four of the five patients who required dialysis at baseline were able to discontinue dialysis.

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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 (± SD) increased from 109 ± 32 x10^9/L at baseline to 169 ± 72 x10^9/L by one week; this effect was maintained through 26 weeks (210 ± 68 x10^9/L), and 2 years (205 ± 46 x10^9/L). When treatment was continued for more than 26 weeks, two additional patients achieved Hematologic Normalization as well as Complete TMA response. Hematologic Normalization and Complete TMA response were maintained by all responders. In aHUS Study 1, responses to Soliris were similar in patients with and without identified mutations in genes encoding complement regulatory factor proteins.

Table 11 summarizes the efficacy results for aHUS Study 1.

Table 11: Efficacy Results for aHUS Study 1

Efficacy Parameter	aHUS Study 1 at 26 wks[1] N = 17	aHUS Study 1 at 2 yrs[2] N = 17
Complete TMA response, n (%)	11 (65)	13 (77)
Median Duration of complete TMA response, weeks (range)	38 (25, 56)	99 (25, 139)
eGFR improvement ≥15 mL/min/1.73 m^2, n (%)	9 (53)	10 (59)
Median duration of eGFR improvement, days (range)	251 (70, 392)	ND
Hematologic normalization, n (%)	13 (76)	15 (88)
Median Duration of hematologic normalization, weeks (range)	37 (25, 62)	99 (25, 145)
TMA event-free status, n (%)	15 (88)	15 (88)
Daily TMA intervention rate, median (range)		
Before eculizumab	0.82 (0.04, 1.52)	0.82 (0.04, 1.52)
On eculizumab treatment	0 (0, 0.31)	0 (0, 0.36)

[1] At data cut-off (September 8, 2010).

[2] At data cut-off (April 20, 2012).

aHUS Sensitive to PE/PI (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 were required to have ADAMTS13 activity level above 5%; observed range of values in the trial were 37%-118%. Seventy percent of patients had an identified complement regulatory factor mutation or auto-antibody. Table 12 summarizes the key baseline clinical and disease-related characteristics of patients enrolled in aHUS Study 2.

Table 12: 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. In aHUS Study 2, the median duration of Soliris therapy was approximately 114 weeks (range: 26 to 129 weeks).

Renal function, as measured by eGFR, was maintained during Soliris therapy. The mean eGFR (± SD) was 31 ± 19 mL/min/1.73m^2 at baseline, and was maintained through 26 weeks (37 ± 21 mL/min/1.73m^2) and 2 years (40 ± 18 mL/min/1.73m^2). No patient required new dialysis with Soliris.

Reduction in terminal complement activity was observed in all patients after the commencement of Soliris. Soliris reduced signs of complement-mediated TMA activity, as shown by an increase in mean platelet counts from baseline to 26 weeks. Platelet counts were maintained at normal levels despite the elimination of PE/PI. The mean platelet count (± SD) was 228 ± 78 x10^9/L at baseline, 233 ± 69 x10^9/L at week 26, and 224 ± 52 x10^9/L at 2 years. When treatment was continued for more than 26 weeks, six additional patients achieved Complete TMA response. Complete TMA Response and Hematologic Normalization were maintained by all responders. In aHUS Study 2, responses to Soliris were similar in patients with and without identified mutations in genes encoding complement regulatory factor proteins.

Table 13 summarizes the efficacy results for aHUS Study 2.

Table 13: Efficacy Results for aHUS Study 2

Efficacy Parameter	aHUS Study 2 at 26 wks[1] N = 20	aHUS Study 2 at 2 yrs[2] N = 20
Complete TMA response, n (%)	5 (25)	11 (55)
Median duration of complete TMA response, weeks (range)	32 (12, 38)	68 (38, 109)
eGFR improvement ≥15 mL/min/1.73 m^2, n (%)	1 (5)	8 (40)
TMA Event-free status n (%)	16 (80)	19 (95)
Daily TMA intervention rate, median (range)		
Before eculizumab	0.23 (0.05, 1.07)	0.23 (0.05, 1.07)
On eculizumab treatment	0	0 (0, 0.01)
Hematologic normalization[4], n (%)	18 (90)	18 (90)
Median duration of hematologic normalization, weeks (range)[3]	38 (22, 52)	114 (33, 125)

[1] At data cut-off (September 8, 2010).

[2] At data cut-off (April 20, 2012).

[3] Calculated at each post-dose day of measurement (excluding Days 1 to 4) using a repeated measurement ANOVA model.

[4] In 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.

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 (± SD) increased from 171 ± 83 x10^9/L at baseline to 233 ±109 x10^9/L after one week of therapy; this effect was maintained through 26 weeks (mean platelet count (± SD) at week 26: 254 ± 79 x10^9/L).

A total of 19 pediatric patients (ages 2 months to 17 years) received Soliris in 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 <18 years of age (n=4). Fifty three percent of pediatric patients had an identified complement regulatory factor mutation or auto-antibody.

Overall, the efficacy results for these pediatric patients appeared consistent with what was observed in patients enrolled in aHUS Studies 1 and 2 (Table 14). No pediatric patient required new dialysis during treatment with Soliris.

Table 14: 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)
Complete TMA response, n (%)	2 (40)	5 (50)	1 (25)	8 (42)
Patients with eGFR improvement ≥ 15 mL/min/1.73 m^2, n (%)[2]	2 (40)	6 (60)	1 (25)	9 (47)
Platelet count normalization, n (%)[1]	4 (80)	10 (100)	3 (75)	17 (89)
Hematologic Normalization, n (%)	2 (40)	5 (50)	1 (25)	8 (42)
Daily TMA intervention rate, median (range)				
Before eculizumab	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)

[1] Platelet count normalization was defined as a platelet count of at least 150,000 X 10^9/L on at least two consecutive measurements spanning a period of at least 4 weeks.

[2] Of the 9 patients who experienced an eGFR improvement of at least 15 mL/min/1.73 m^2, one received dialysis throughout the study period and another received Soliris as prophylaxis following renal allograft transplantation.

Adult Patients with aHUS (aHUS Study 4)

aHUS Study 4 enrolled patients who displayed signs of thrombotic microangiopathy (TMA). In order to qualify for enrollment, patients were required to have a platelet count < lower limit of normal range (LLN), evidence of hemolysis such as an elevation in serum LDH, and serum creatinine above the upper limits of normal, without the need for chronic dialysis. The median patient age was 35 (range: 18 to 80 years). All patients enrolled in aHUS Study 4 were required to have ADAMTS13 activity level above 5%; observed range of values in the trial were 28%-116%. Fifty-one percent of patients had an identified complement regulatory factor mutation or auto-antibody. A total of 35 patients received PE/PI prior to eculizumab. Table 15 summarizes the key baseline clinical and disease-related characteristics of patients enrolled in aHUS Study 4.

Table 15: Baseline Characteristics of Patients Enrolled in aHUS Study 4

Parameter	aHUS Study 4 N = 41
Time from aHUS diagnosis until start of study drug in months, median (range)	0.79 (0.03 – 311)
Time from current clinical TMA manifestation until first study dose in months, median (range)	0.52 (0.03-19)
Baseline platelet count (× 10^9/L), median (range)	125 (16 – 332)
Baseline LDH (U/L), median (range)	375 (131 – 3318)

Patients in aHUS Study 4 received Soliris for a minimum of 26 weeks. In aHUS Study 4, the median duration of Soliris therapy was approximately 50 weeks (range: 13 weeks to 86 weeks).

Renal function, as measured by eGFR, was improved during Soliris therapy. The mean eGFR (± SD) increased from 17 ± 12 mL/min/1.73m^2 at baseline to 47 ± 24 mL/min/1.73m^2 by 26 weeks. Twenty of the 24 patients who required dialysis at study baseline were able to discontinue dialysis during Soliris treatment.

Reduction in terminal complement activity and an increase in platelet count relative to baseline were observed after commencement of Soliris. Soliris reduced signs of complement-mediated TMA activity, as shown by an increase in mean platelet counts from baseline to 26 weeks. In aHUS Study 4, mean platelet count (± SD) increased from 119 ± 66 x10^9/L at baseline to 200 ± 84 x10^9/L by one week; this effect was maintained through 26 weeks (mean platelet count (± SD) at week 26: 252 ± 70 x10^9/L). In aHUS Study 4, responses to Soliris were similar in patients with and without identified mutations in genes encoding complement regulatory factor proteins or auto-antibodies to factor H.

Table 16 summarizes the efficacy results for aHUS Study 4.

Table 16: Efficacy Results for aHUS Study 4

Efficacy Parameter	aHUS Study 4 (N = 41)
Complete TMA response, n (%),	23 (56),
95% CI	40,72
Median duration of complete TMA response, weeks (range)	42 (6, 75)
Patients with eGFR improvement ≥ 15 mL/min/1.73 m2, n (%)	22 (54)
Hematologic Normalization, n (%)	36 (88)
Median duration of hematologic normalization, weeks (range)	46 (10, 75)
TMA Event-free Status, n (%)	37 (90)
Daily TMA Intervention Rate, median (range)	
Before eculizumab	0.63 (0, 1.38)
On eculizumab treatment	0 (0, 0.58)

Pediatric and Adolescent Patients with aHUS (aHUS Study 5)

aHUS Study 5 enrolled patients who were required to have a platelet count < lower limit of normal range (LLN), evidence of hemolysis such as an elevation in serum LDH above the upper limits of normal, serum creatinine level ≥97 percentile for age without the need for chronic dialysis. The median patient age was 6.5 (range: 5 months to 17 years). Patients enrolled in aHUS Study 5 were required to have ADAMTS13 activity level above 5%;observed range of values in the trial were 38%-121%. Fifty percent of patients had an identified complement regulatory factor mutation or auto-antibody. A total of 10 patients received PE/PI prior to eculizumab. Table 17 summarizes the key baseline clinical and disease-related characteristics of patients enrolled in aHUS Study 5.

Table 17: Baseline Characteristics of Patients Enrolled in aHUS Study 5

Parameter	Patients 1 month to <12 years (N = 18)	All Patients (N = 22)
Time from aHUS diagnosis until start of study drug in months, median (range)	0.51 (0.03 – 58)	0.56 (0.03-191)
Time from current clinical TMA manifestation until first study dose in months, median (range)	0.23 (0.03 – 4)	0.2 (0.03-4)
Baseline platelet count (x 10^9/L), median (range)	110 (19-146)	91 (19-146)
Baseline LDH (U/L) median (range)	1510 (282-7164)	1244 (282-7164)

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Patients in aHUS Study 5 received Soliris for a minimum of 26 weeks. In aHUS Study 5, the median duration of Soliris therapy was approximately 44 weeks (range: 1 dose to 88 weeks).

Renal function, as measured by eGFR, was improved during Soliris therapy. The mean eGFR (± SD) increased from 33 ± 30 mL/min/1.73m^2 at baseline to 98 ± 44 mL/min/1.73m^2 by 26 weeks. Among the 20 patients with a CKD stage ≥2 at baseline, 17 (85%) achieved a CKD improvement of ≥1 stage. Among the 16 patients ages 1 month to <12 years with a CKD stage ≥2 at baseline, 14 (88%) achieved a CKD improvement by ≥1 stage. Nine of the 11 patients who required dialysis at study baseline were able to discontinue dialysis during Soliris treatment. Responses were observed across all ages from 5 months to 17 years of age.

Reduction in terminal complement activity was observed in all patients after commencement of Soliris. Soliris reduced signs of complement-mediated TMA activity, as shown by an increase in mean platelet counts from baseline to 26 weeks. The mean platelet count (± SD) increased from 88 ± 42 x10^9/L at baseline to 281 ± 123 x10^9/L by one week; this effect was maintained through 26 weeks (mean platelet count (±SD) at week 26: 293 ± 106 x10^9/L). In aHUS Study 5, responses to Soliris were similar in patients with and without identified mutations in genes encoding complement regulatory factor proteins or auto-antibodies to factor H.

Table 18 summarizes the efficacy results for aHUS Study 5.

Table 18: Efficacy Results for aHUS Study 5

Efficacy Parameter	Patients 1 month to <12 years (N = 18)	All Patients (N = 22)
Complete TMA response, n (%)	11 (61)	14 (64)
95% CI	36, 83	41, 83
Median Duration of complete TMA response, weeks (range)[1]	40 (14, 77)	37 (14, 77)
eGFR improvement ≥15 mL/min/ 1.73•m^2•n (%)	16 (89)	19 (86)
Complete Hematologic Normalization, n (%)	14 (78)	18 (82)
Median Duration of complete hematologic normalization, weeks (range)	38 (14, 77)	38 (14, 77)
TMA Event-Free Status, n (%)	17 (94)	21 (95)
Daily TMA Intervention rate, median (range)		
Before eculizumab treatment	0.2 (0, 1.7)	0.4 (0, 1.7)
On eculizumab treatment	0 (0, 0.01)	0 (0, 0.01)

[1] through data cutoff (October 12, 2012).

16 HOW SUPPLIED/STORAGE AND HANDLING

Soliris (eculizumab) is supplied as 300 mg single-dose vials containing 30 mL of 10 mg/mL sterile, preservative-free Soliris solution per vial.

Store Soliris vials in the original carton until time of use under refrigerated conditions at 2-8° C (36-46° F) and protected from light. Soliris vials may be held in the original carton at controlled room temperature (not more than 25° C/77° F) for only a single period up to 3 days.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

Advise the patient to read FDA-approved patient labeling (Medication Guide).

Meningococcal Infection

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.

Inform patients that they are required to receive 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. Inform patients that vaccination may not prevent meningococcal infection *[see Warnings and Precautions (5.1)]*.

Signs and Symptoms of Meningococcal Infection

Inform patients about the signs and symptoms of meningococcal infection, and strongly advise patients 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
- fever and a rash
- confusion
- muscle aches with flu-like symptoms
- eyes sensitive to light

Inform patients 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.

Other Infections

Inform patients that there may be an increased risk of other types of infections, particularly those due to encapsulated bacteria. Additionally, Aspergillus infections have occured in immunocompromised and neutropenic patients. Inform parents or caregivers of children receiving Soliris for the treatment of aHUS that their child should be vaccinated against *Streptococcus pneumoniae* and *Haemophilus influenza* type b (Hib) according to current medical guidelines.

Discontinuation

Inform patients with PNH 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. Inform patients with aHUS 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. Inform patients who discontinue Soliris 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.
100 College Street
New Haven, CT 06510 USA

US License Number 1743

This product, or its use, may be covered by one or more US patents, including US Patent No. 6,355,245 in addition to others including patents pending.

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 chance 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 meningococcal vaccination at least 2 weeks before your first dose of Soliris unless you have already had this vaccine. If your doctor decided that urgent treatment with Soliris is needed, you should receive meningococcal vaccination as soon as possible.**
2. **If you had a meningococcal vaccine in the past, you might need additional vaccination before starting Soliris. Your doctor will decide if you need additional meningococcal vaccination.**
3. **Meningococcal vaccines do 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**
   - **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 *Haemophilis 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 medications 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 in adults and 1-4 hours in pediatric patients. 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 cell count
  - 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?"

- 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:

- headache
- diarrhea
- hypertension
- common cold (upper respiratory infection)
- abdominal pain
- vomiting
- nasopharyngitis
- anemia
- cough
- peripheral edema
- nausea
- urinary tract infections
- pyrexia

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., 100 College Street, New Haven, CT 06510 USA.

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Declaration by Dr. Michael Treuheit

I, Michael Treuheit, Ph.D., declare that:

1. I am and have been employed by Amgen Inc. since February, 1993. Since 2006, I have held the title of Director Process Development. In this role, I am responsible/oversee formulation development of large molecule therapeutics, such as cytokines, antibodies, and antibody fragments. During my career, I have been directly involved in the formulation of at least 30 clinically tested and/or marketed therapeutic antibodies. A current copy of my CV is provided as **Appendix D**.

2. A study was conducted to demonstrate the stability of eculizumab over a range of concentrations. Eculizumab was formulated at 2 mg/ml (2PBST22), 5 mg/ml (5PBST22), 10 mg/ml (10PBST22), 30 mg/ml (30PBST22), and 50 mg/ml (50PBST22) in 10 mM Na Phosphate, 150 mM NaCl, 0.022 % polysorbate 80 at pH 7.0. Soliris® (eculizumab) is 10 mg/ml eculizumab formulated in 10 mM Na Phosphate, 150 mM NaCl, 0.022 % polysorbate 80 at pH 7.0. The EMA label indicates Soliris should be stored between 2-8°C.

3. Like Soliris®, the eculizumab formulations used in the study were sterile and preservative free.

4. The stability of the 2 mg/ml, 5 mg/ml, 10 mg/ml, 30 mg/ml, and 50 mg/ml eculizumab formulations was monitored over a period of 13 weeks at storage conditions of (4°C) and at elevated temperature (25°C). Storage at the elevated temperature is used to evaluate the product specific routes of degradation in an accelerated manner as compared to recommended storage conditions. Physical and chemical stability were analyzed by size-exclusion ultra high-performance liquid chromatography (SE-UHPLC), anion exchange high-performance liquid chromatography (AEX-HPLC), and reduce sodium dodecyl sulfate capillary electrophoresis (rCE-SDS).

5. Aggregation of the antibody results in the formation of high molecular weight (HMW) species that can be visualized by SE-UHPLC. A large degree of HMW formation is a sign of low long-term stability. All of the eculizumab solutions stored at 4°C and 25°C were sufficiently stable throughout the study, i.e., there was no significant loss in main peak for any of the protein concentrations examined. The results are summarized in **Appendix A**.

6. The storage of antibodies in solution can result in chemical degradation, leading to the formation of acidic species (e.g. by deamidation of asparagine residues), which can be detected by AEX-HPLC. A high degree of acidic species is a sign of low chemical stability. Analysis of the eculizumab solutions stored under label conditions and the elevated temperature showed that all tested antibody concentrations were essentially equivalent. At the elevated temperature, all showed some chemical degradation, which increases over time upon storage, but there is no significant difference in chemical stability between protein concentrations. The results are summarized in **Appendix B**.

7. rCE-SDS offers high resolution separation of most types of chemically or structurally altered species from the main (intact) species for both heavy chain (HC) and light chain (LC) of the antibody. Analysis of the eculizumab solutions by rCE-SDS shows that a detectable decrease of the main species (HC+LC) occurs at both tested temperatures for all samples. However, in agreement with the AEX-HPLC measurements, the antibody solutions were equivalent at the recommended storage conditions and elevated temperature. The results are summarized in **Appendix C**.

8. The results of the SE-UHPLC, AEX-HPLC, and rCE-SDS analyses demonstrate eculizumab is stable at the range of concentration tested, 2-50 mg/ml, and there is no significant difference in stability amongst the concentrations tested. Thus, the 10 mg/ml formulation does not demonstrate superior stability compared to the less- or more-concentrated formulations of eculizumab.

I certify that the above statements are correct to the best of my knowledge and belief.

Amgen Thousand Oaks, CA, Aug 3, 2017

Place and Date

Ph.D.

Dr. Michael Treuheit

Attachments
Appendices A-D

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CURRICULUM VITAE

Michael Treuheit, Ph.D.

Education

Doctor of Philosophy in Chemistry, October 1990, University of Cincinnati.
Thesis Title: *Disease-Coupled Specificity In The Glycan Structures of Orosomucoid.*

Bachelor of Science in Biology, June 1980, University of Cincinnati.

Work Experience

Amgen, Inc., 1993-present

Director of Drug Product Formulation Technology in the Department of Product Development leading a group of approximately 30 scientists (15 at the PhD. Level). The group has responsibility for developing FIH and commercial formulations, and for the CMC sections of our large molecule MA's.
Notable past accomplishments include the submission of the Development Pharmaceutics Section in the BLA's for Neulasta , Palifermin and Denosumab along with a variety of IND's and INDa's for our pipeline molecules

University of Cincinnati, College of Medicine, Department of Pharmacology and Cell Biophysics. Postdoctoral Fellowship in the Protein Chemistry Core Laboratory of Dr. Terence L. Kirley, 1990 to 1993.

University of Cincinnati, Department of Chemistry Teaching Assistant, 1985 to 1990.
Taught freshman, Bio-Organic and Biochemistry laboratory and selected lecture courses.

The Christ Hospital, Research Technologist in the Tumor Research Laboratory, experienced in tissue culture techniques (Human Tumor Clonogenic Assay), 1975 to 1989.

Abstracts and Presentations

Kim, S.B., Distler, C. and Treuheit, M. Human Tumor Clonogenic Assay (HTCA) In Patients With Metastatic Adenocarcinoma, Primary Unknown. Presented at the Annual meeting, Central Society for Clinical Research, Chicago, 1984.

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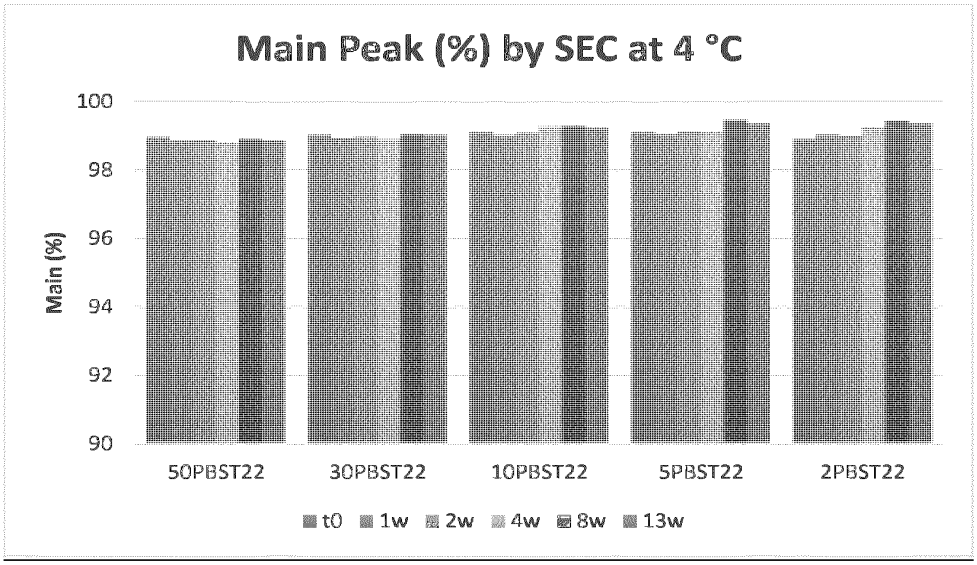
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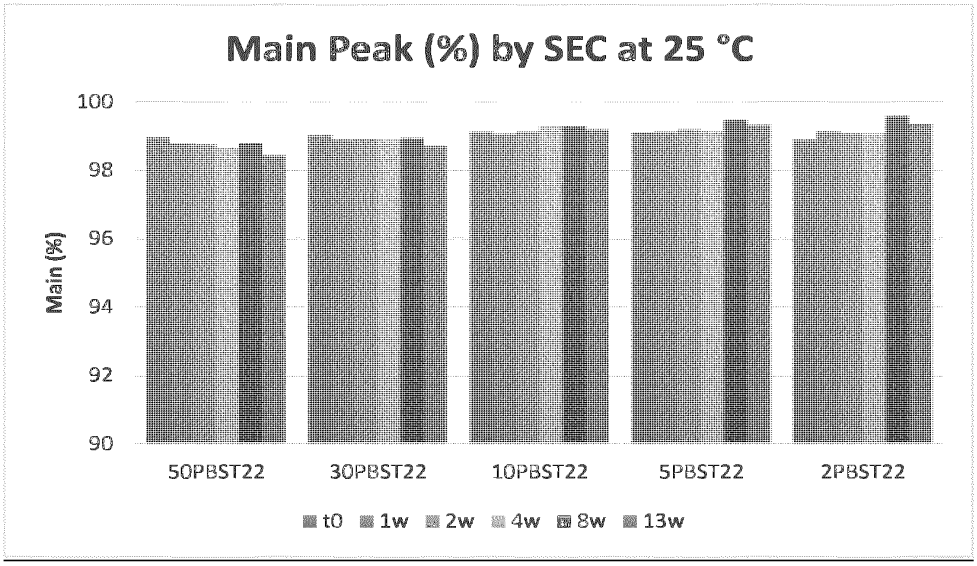
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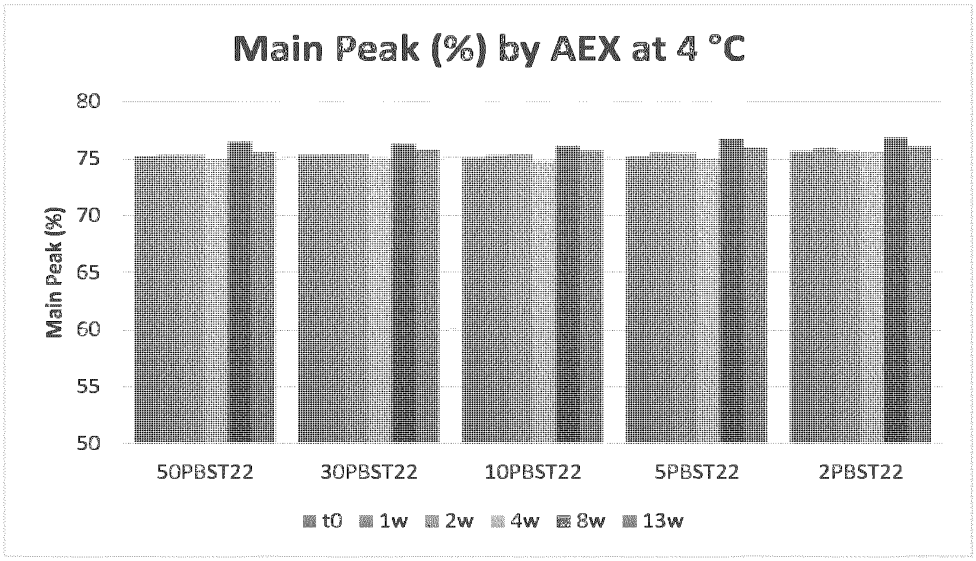
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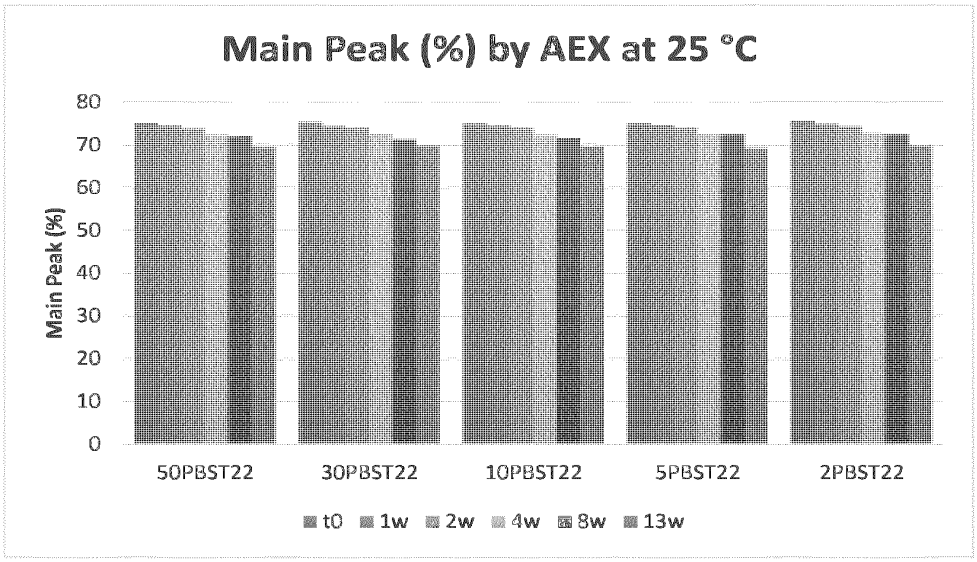




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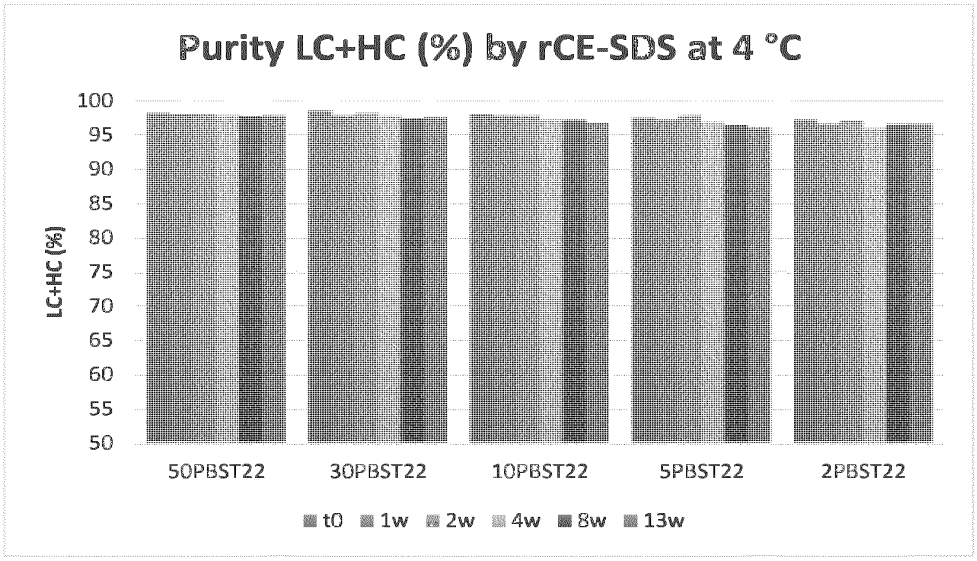
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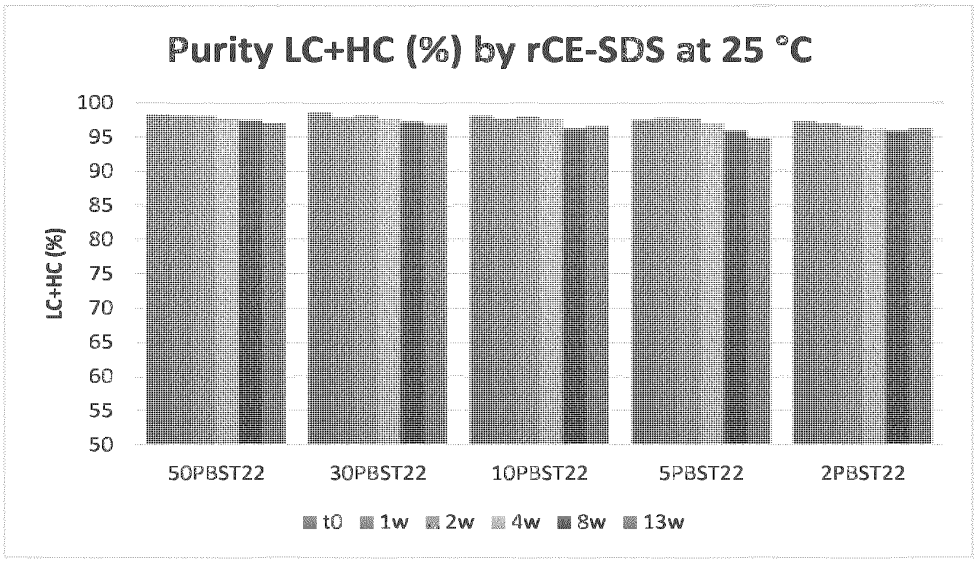




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Appendix C





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XP001538426

ADVANCES IN HEMATOLOGY

Current Developments in the Management of Hematologic Disorders

Section Editor: Craig M. Kessler, MD

Eculizumab for the Treatment of Paroxysmal Nocturnal Hemoglobinuria

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H&O Can you give a short background on the standard of care for paroxysmal nocturnal hemoglobinuria before the advent of eculizumab?

AH Until recently, the treatment for paroxysmal nocturnal hemoglobinuria (PNH) was predominantly supportive. Red blood cell (RBC) transfusions were administered when appropriate, and warfarin or other anticoagulation therapy was given following a thrombosis. In the UK we practice primary prophylaxis with anticoagulant therapy depending on the neutrophil PNH clone size. Other therapies, such as corticosteroids or androgen therapy, have also been used, but less commonly and with efficacy in only a few patients; the mechanism of action behind these treatments is unclear. Stem cell transplantation has also been used in the past, but of course this approach is associated with serious complications. It has been difficult to develop an evidence base for treatment because of the heterogeneous nature and rarity of this disease. Before eculizumab (Alexion), treatment for PNH was limited to alleviating symptoms and improving patient quality of life. Eculizumab is a monoclonal antibody that blocks the complement system at protein C5.

H&O What is the rationale for the development of eculizumab for the treatment of PNH?

AH We know the hemolysis in PNH is complement-mediated, thus this drug should be effective in preventing hemolysis. The pathogenesis of PNH is known to be due to complement sensitivity of RBCs against complement attack. RBCs in PNH have lost their protective proteins (eg, CD55 and CD59) that would normally prevent complement-mediated lysis. It was hypothesized that if the complement attack on the RBCs could be blocked, the hemolysis in PNH and its associated symptoms could be prevented. Based on patients who have inherited complement deficiencies, it appears that patients with deficiencies in complement proteins C5–C9 suffer few clinical consequences compared to those with deficiencies in the complement proteins before C5. Therefore, C5 seemed to be a logical target for blocking the complement cascade. Eculizumab was developed for the purpose of blocking the complement system safely and without severe complications.

H&O Eculizumab has been evaluated for the treatment of rheumatoid arthritis, systemic lupus erythematosus, and other autoimmune conditions. Is there a similarity between these conditions and PNH that spurred investigators to evaluate eculizumab in PNH?

AH The research strategy has been to develop therapeutic approaches that block complement activity, which contributes to a variety of disorders. There is some evidence that some components of autoimmune disorders such as rheumatoid arthritis and systemic lupus erythematosus are complement-mediated. In PNH, however, almost all of the clinical symptoms are due to the uncontrolled activity of complement. Therefore, whereas there are many possible disorders in which complement blockade might have some efficacy, PNH is the disorder in which we had little doubt that eculizumab would be effective.

H&O What were some of the initial findings with eculizumab in PNH?

AH In May 2002, we began the pilot study of eculizumab in PNH. Patients enrolled had been having regular transfusions, and most exhibited dark urine (a sign of intravascular hemolysis) and severe symptoms of PNH such as difficulty swallowing (dysphagia) and abdominal pain. Men complained of erectile dysfunction. Additionally, they had biochemical parameters indicative of active, ongoing intravascular hemolysis. We found that just 1 infusion of the drug caused a resolution of the

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dark urine by the next morning. Other symptoms, such as abdominal pain and difficulty in swallowing, resolved quickly. Of the 6 male patients who had experienced erectile dysfunction, 5 experienced complete resolution of this symptom. The patients felt better quickly and, in terms of biochemical parameters, we found quick improvements in lactate dehydrogenase levels as well.

H&O What were the next steps after this initial study?

AH The pilot study was initially planned to be 12 weeks in duration, but at the end of the 12 weeks, all patients wanted to continue with eculizumab therapy. The extension phase of the trial commenced and is still continuing with 10 of the 11 patients. Patients have experienced a dramatic improvement in their quality of life and generally prefer 2 weekly infusions of eculizumab rather than their previous treatment, which might have been, for example, 4 weekly blood transfusions. Some patients have become transfusion-independent.

H&O What side effects are associated with eculizumab?

AH The side effects associated with eculizumab have been very minimal. One patient in the pilot study had a reaction to the initial infusion, consisting of shivering and fever. She required observation only and has tolerated every infusion after that extremely well with no further reactions; she is still on the drug more than 3 years later. No other patients experienced any infusion-related reactions. The only minor side effect has been headache after the first or second infusions in some of the patients, but otherwise toxicity has not been a problem. There is a theoretical risk of meningitis, but this has not been a major issue to date.

H&O Is there any sense about whether eculizumab treats all patients equally or are there variations between patients that might change the efficacy of the agent?

AH We found that patients who have the classic purely hemolytic PNH respond better to the drug than those who have a hypoplastic bone marrow. The reason for this difference is simply that marrow failure can sometimes contribute more significantly than hemolysis to anemia, in which case, although the hemolysis will be blocked by eculizumab, marrow failure will most likely still lead to anemia and therefore continuing transfusions will be required. Certainly any patient with classical PNH who is hemolyzing frequently is a good candidate for eculizumab therapy. This agent would also be appropriate for patients with biochemical and clinical evidence of hemolysis because their quality of life can be improved.

H&O What is the clinical trial status of eculizumab?

AH Two phase III trials opened worldwide in the last year: TRIUMPH and SHEPHERD. The first opened about a year ago and the second opened earlier this year. The trials are now closed to recruitment, and we are eagerly awaiting the results. If the results of this trial are positive, I would expect that applications for product licenses would be made relatively quickly.

H&O Does eculizumab appear to have any effect on thrombosis, the most significant morbidity associated with PNH?

AH Although at the moment we have no evidence that eculizumab will prevent thrombosis, it is reasonable to hypothesize that thrombosis may be prevented by this terminal complement blockade through its mechanism of action. Theories for the causes of thrombosis in PNH include activation of PNH platelets by complement attack and nitric oxide depletion through free hemoglobin scavenging. Eculizumab should prevent both consequences. This possibility will be very interesting to examine in the future. In the pilot study there was no evidence of thromboses in any of the patients.

Suggested Reading

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Letter accompanying subsequently filed items

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The document(s) listed below is (are) subsequently filed documents pertaining to the following application:

Application number	11001632.6
Applicant's or representative's reference	AMG17203OP

	Description of document	Original file name	Assigned file name
1	Maintenance of the application	AMG17203OP_Request for D6.pdf	MEPA-1.pdf

	Payment	
1	Mode of payment	Not specified

Signatures

Place:	**Munich, Germany**
Date:	**08 August 2017**
Signed by:	**Hans Doerries 13689**
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August 7, 2017

European patent 2 359 834 (Application No. 11 001 632.6)
Title: Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement - EP 2 359 834
Proprietor: Alexion Pharmaceuticals, Inc.
Opponent: Amgen Inc.
Our Ref.: AMG17203OP

URGENT

With respect to the Notice of Opposition as filed on August 4, 2017

We hereby kindly request provision of a document cited during the prosecution of the above identified European patent at the earliest opportunity by either Telefax ((+49 89 210296-33) or e-mail to info@df-mp.com.

The document is **XP001538426**; HILL ANITA, "Eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria", *CLINICAL ADVANCES IN HEMATOLOGY & ONCOLOGY*, MILLENNIUM MEDICAL PUB., NEW YORK, NY, US, (20051101), vol. 3, no. 11, ISSN 1543-0790, pages 849 – 850.

The document is cited in the European Search report, dated July 12, 2011, but was not made available via Espacenet. We are having difficulties to retrieve this document via other sources.

Thank you very much!

Respectfully,

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Submission in opposition proceedings

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- representing the opponent(s):	Amgen, Inc.
Opponent/representative's reference	AMG17203OP

The information given below is pertaining to the following patent in opposition proceedings:

Patent No.	EP2359834
Application No.	EP11001632.6
Title of the invention	Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement
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 - Submission regarding document D6	AMG17203OP_Filing_D6.pdf	OTHER-1.pdf

Evidence filed subsequently:

D6	Non-patent literature - article	Hill, "Eculizumab for the Treatment of Paroxysmal Nocturnal Hemoglobinuria" Clinical Advances in Hematology & Oncology, 2005 original file name: D6 - Hill .pdf attached as: Published-Evidence-1.pdf

Signatures

AMG17203OP

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Page 6321

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Date:	**14 August 2017**
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With respect to the Notice of Opposition as filed on August 4, 2017

We herewith provide the Opposition Division with a copy of the document **D6**.

The document is cited in the European Search report, dated July 12, 2011, but was not made available via Espacenet. We have had difficulties to retrieve this document via other sources.

The document **XP001538426**; HILL ANITA, "Eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria", *CLINICAL ADVANCES IN HEMATOLOGY & ONCOLOGY*, MILLENNIUM MEDICAL PUB., NEW YORK, NY, US, (20051101), vol. 3, no. 11, ISSN 1543-0790, pages 849 – 850, is enclosed herewith.

Respectfully,

Solveig Moré

Dr. Solveig Moré
European Patent Attorney

UD/MO/CB

Enclosure
Document D6

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Partnerschaftsregister des AG München
PR-Nr. 1278

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XP001538428

ADVANCES IN HEMATOLOGY

Current Developments in the Management of Hematologic Disorders

Section Editor: Craig M. Kessler, MD

Eculizumab for the Treatment of Paroxysmal Nocturnal Hemoglobinuria

Anita Hill, MBChB(Hons), MRCP, DipRCPath
Clinical Research Fellow
Department of Haematology
Leeds Teaching Hospitals NHS Trust

H&O Can you give a short background on the standard of care for paroxysmal nocturnal hemoglobinuria before the advent of eculizumab?

AH Until recently, the treatment for paroxysmal nocturnal hemoglobinuria (PNH) was predominantly supportive. Red blood cell (RBC) transfusions were administered when appropriate, and warfarin or other anticoagulation therapy was given following a thrombosis. In the UK we practice primary prophylaxis with anticoagulant therapy depending on the neutrophil PNH clone size. Other therapies, such as corticosteroids or androgen therapy, have also been used, but less commonly and with efficacy in only a few patients; the mechanism of action behind these treatments is unclear. Stem cell transplantation has also been used in the past, but of course this approach is associated with serious complications. It has been difficult to develop an evidence base for treatment because of the heterogeneous nature and rarity of this disease. Before eculizumab (Alexion), treatment for PNH was limited to alleviating symptoms and improving patient quality of life. Eculizumab is a monoclonal antibody that blocks the complement system at protein C5.

H&O What is the rationale for the development of eculizumab for the treatment of PNH?

AH We know the hemolysis in PNH is complement-mediated, thus this drug should be effective in preventing hemolysis. The pathogenesis of PNH is known to be due to complement sensitivity of RBCs against complement attack. RBCs in PNH have lost their protective proteins (eg, CD55 and CD59) that would normally prevent complement-mediated lysis. It was hypothesized that if the complement attack on the RBCs could be blocked, the hemolysis in PNH and its associated symptoms could be prevented. Based on patients who have inherited complement deficiencies, it appears that patients with deficiencies in complement proteins C5–C9 suffer few clinical consequences compared to those with deficiencies in the complement proteins before C5. Therefore, C5 seemed to be a logical target for blocking the complement cascade. Eculizumab was developed for the purpose of blocking the complement system safely and without severe complications.

H&O Eculizumab has been evaluated for the treatment of rheumatoid arthritis, systemic lupus erythematosus, and other autoimmune conditions. Is there a similarity between these conditions and PNH that spurred investigators to evaluate eculizumab in PNH?

AH The research strategy has been to develop therapeutic approaches that block complement activity, which contributes to a variety of disorders. There is some evidence that some components of autoimmune disorders such as rheumatoid arthritis and systemic lupus erythematosus are complement-mediated. In PNH, however, almost all of the clinical symptoms are due to the uncontrolled activity of complement. Therefore, whereas there are many possible disorders in which complement blockade might have some efficacy, PNH is the disorder in which we had little doubt that eculizumab would be effective.

H&O What were some of the initial findings with eculizumab in PNH?

AH In May 2002, we began the pilot study of eculizumab in PNH. Patients enrolled had been having regular transfusions, and most exhibited dark urine (a sign of intravascular hemolysis) and severe symptoms of PNH such as difficulty swallowing (dysphagia) and abdominal pain. Men complained of erectile dysfunction. Additionally, they had biochemical parameters indicative of active, ongoing intravascular hemolysis. We found that just 1 infusion of the drug caused a resolution of the

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dark urine by the next morning. Other symptoms, such as abdominal pain and difficulty in swallowing, resolved quickly. Of the 6 male patients who had experienced erectile dysfunction, 5 experienced complete resolution of this symptom. The patients felt better quickly and, in terms of biochemical parameters, we found quick improvements in lactate dehydrogenase levels as well.

H&O What were the next steps after this initial study?

AH The pilot study was initially planned to be 12 weeks in duration, but at the end of the 12 weeks, all patients wanted to continue with eculizumab therapy. The extension phase of the trial commenced and is still continuing with 10 of the 11 patients. Patients have experienced a dramatic improvement in their quality of life and generally prefer 2 weekly infusions of eculizumab rather than their previous treatment, which might have been, for example, 4 weekly blood transfusions. Some patients have become transfusion-independent.

H&O What side effects are associated with eculizumab?

AH The side effects associated with eculizumab have been very minimal. One patient in the pilot study had a reaction to the initial infusion, consisting of shivering and fever. She required observation only and has tolerated every infusion after that extremely well with no further reactions; she is still on the drug more than 3 years later. No other patients experienced any infusion-related reactions. The only minor side effect has been headache after the first or second infusions in some of the patients, but otherwise toxicity has not been a problem. There is a theoretical risk of meningitis, but this has not been a major issue to date.

H&O Is there any sense about whether eculizumab treats all patients equally or are there variations between patients that might change the efficacy of the agent?

AH We found that patients who have the classic purely hemolytic PNH respond better to the drug than those who have a hypoplastic bone marrow. The reason for this difference is simply that marrow failure can sometimes contribute more significantly than hemolysis to anemia, in which case, although the hemolysis will be blocked by eculizumab, marrow failure will most likely still lead to anemia and therefore continuing transfusions will be required. Certainly any patient with classical PNH who is hemolyzing frequently is a good candidate for eculizumab therapy. This agent would also be appropriate for patients with biochemical and clinical evidence of hemolysis because their quality of life can be improved.

H&O What is the clinical trial status of eculizumab?

AH Two phase III trials opened worldwide in the last year: TRIUMPH and SHEPHERD. The first opened about a year ago and the second opened earlier this year. The trials are now closed to recruitment, and we are eagerly awaiting the results. If the results of this trial are positive, I would expect that applications for product licenses would be made relatively quickly.

H&O Does eculizumab appear to have any effect on thrombosis, the most significant morbidity associated with PNH?

AH Although at the moment we have no evidence that eculizumab will prevent thrombosis, it is reasonable to hypothesize that thrombosis may be prevented by this terminal complement blockade through its mechanism of action. Theories for the causes of thrombosis in PNH include activation of PNH platelets by complement attack and nitric oxide depletion through free hemoglobin scavenging. Eculizumab should prevent both consequences. This possibility will be very interesting to examine in the future. In the pilot study there was no evidence of thromboses in any of the patients.

Suggested Reading

Hill A, Hillmen P, Richards SJ, et al. Sustained response and long-term safety of eculizumab in paroxysmal nocturnal hemoglobinuria. *Blood*. 2005;106:2559-2565.

Hillmen P, Hall C, Marsh JC, et al. Effect of eculizumab on hemolysis and transfusion requirements in patients with paroxysmal nocturnal hemoglobinuria. *N Engl J Med*. 2004;350:552-559.

Parker C, Omine M, Richards S, et al. Diagnosis and management of paroxysmal nocturnal hemoglobinuria. *Blood*. 2005; [Epub ahead of print]

Peggi A, Negrini S, Zocchi MR, et al. Patients with paroxysmal nocturnal hemoglobinuria have a high frequency of peripheral-blood T cells expressing activating isoforms of inhibiting superfamily receptors. *Blood*. 2005;106:2399-2408.

Young NS. Paroxysmal nocturnal hemoglobinuria: current issues in pathophysiology and treatment. *Curr Hematol Rep*. 2005;4:103-109.

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European Patent Office
Office européen des brevets

European Patent Office
80298 MUNICH
GERMANY

Questions about this communication ?
Contact Customer Services at www.epo.org/contact

Dörries, Hans Ulrich
df-mp
Fünf Höfe
Theatinerstrasse 16
80333 München
ALLEMAGNE

Date
13.09.2017

Reference	Application No./Patent No.
AMG17203OP OPPO 01	11001632.6 - 1466 / 2359834
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

Communication of further notices of opposition pursuant to Rule 79(2) EPC

You are hereby informed pursuant to Rule 79(2) EPC of further notices of opposition, which are also available via the European Patent Register at **www.epo.org/register**. Should you wish to receive any patent 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).

If oral proceedings are to take place, parties are advised to check the electronic file via the European Patent Register in advance of the hearing to ensure they are in possession of all relevant documents.

A copy of the communication pursuant to Rule 79(1) EPC sent to the proprietor of the patent is also enclosed for your information.

The observations of the patent proprietor on the notice of opposition will be communicated to the opponents 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 continued. Your attention is drawn to Article 114(2) EPC.

For the Opposition Division

Europäisches Patentamt • European Patent Office • Office européen des brevets

Enclosures: copy of the communication pursuant to Rule 79(1) EPC (Form 2317A)

O2 : Hoffmann-Eitle with cited documents - non patent literature -.

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European Patent Office
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European Patent Office
80298 MUNICH
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Questions about this communication ?
Contact Customer Services at www.epo.org/contact

Vossius & Partner
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ALLEMAGNE

Date
13.09.2017

Reference	Application No./Patent No.
P2913 EP/1	11001632.6 - 1466 / 2359834
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

Communication of notices of opposition (R. 79(1) EPC)

Notices of opposition have been filed within the opposition period by:

01.
Amgen Inc.
One Amgen Center Drive
Thousand Oaks, CA 91320-1799
ETATS-UNIS D'AMERIQUE
02.
Hoffmann Eitle
Patent- und Rechtsanwälte PartmbB
Arabellastrasse 30
81925 München
ALLEMAGNE

The notices of opposition indicated above have 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

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Registered letter

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European Patent Office
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EPO - Munich
101
09. Aug. 2017

Zur Kasse
185

Notice of opposition to a European patent

I. Patent opposed

Patent No.	2 359 834
Application No.	11 001 632.6
Date of mention of the grant in the European Patent Bulletin (Art. 97(3), Art. 99(1) EPC)	
Title of the invention	Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement

II. Proprietor of the patent
first named in the patent specification

	Alexion Pharmaceuticals, Inc.
Opponent's or representative's reference (max. 15 keystrokes)	198 441 u6/ne

III. Opponent

Name	Hoffmann Eitle Patent- und Rechtsanwälte PartmbB
Address	Arabellastr. 30 81925 Munich (DE)
State of residence or of principal place of business	DE
Nationality	
Telephone/Fax	
Multiple opponents (see additional sheet)	☐

IV. Authorisation

1. Representative (name only one representative or name of association of representatives to whom notification is to be made)	Hoffmann Eitle Patent- und Rechtsanwälte PartmbB
Address of place of business	Arabellastraße 30 81925 Munich Germany Association No. 151
Telephone/Fax	089 / 92409-0 | 089 / 918356
Additional representative(s) on additional sheet/see authorisation	☐

Opponent's reference
198 441 u6/ne

EPO 2300.1 12.07

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2. Name(s) of employee(s) of the opponent authorised to act in these opposition proceedings under Art. 133(3) EPC

Authorisation(s) to 1./2.

not considered necessary [x]

has/have been registered under No. [ ]

is/are enclosed [ ]

V. Opposition is filed against

- the patent as a whole [x]
- 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) [x]
- patentability is excluded on other grounds, i.e. Article [ ] Art.

(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 a separate sheet (annex 1) [X]

VIII. Other requests:

Oral proceedings if the maintenance of the patent amended or unamended is considered.

Opponent's reference
198 441 u6/ne

IX. Evidence presented

Evidence is enclosed [x]

will be filed at a later date [ ]

A. Publications:

1

Particular relevance (page, column, line, fig.):

B: EP 2 359 834 B1 (opposed patent)

2

Particular relevance (page, column, line, fig.):

A: WO 2007/106585 A1 (application as filed)

3

Particular relevance (page, column, line, fig.):

C: US 60/783,070 (priority application)

4

Particular relevance (page, column, line, fig.):

D1: US 2005/0191298 A1

5

Particular relevance (page, column, line, fig.):

D3: Hillmen et al., N Engl. J Med 2004; 350: 552-559

6

Particular relevance (page, column, line, fig.):

D7: US 6,355,245 B1

Continued on additional sheet [x]

B. Other evidence

Continued on additional sheet [ ]

Opponent's reference
198 441 u6/ne

EPO 2300.3 12.07

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X. Payment of the opposition fee is made

- as indicated in the enclosed voucher for payment of fees and costs (EPO Form 1010) [x]
- via EPO Online Services [ ]

XI. List of documents

Enclosure No.

0 Form for notice of opposition [X]

1 Facts (see VII.) [X]

2 Copies of documents presented as evidence (see IX.)

a Publications [x]

b Other documents [ ]

3 Signed authorisation(s) (see IV.) [ ]

4 Voucher for payment of fees and costs (see X.) [x]

5 Cheque [ ]

6 Additional sheet(s) [x] Number of sheets 1

7 Other [x]

Please specify here:

- receipt form

XII. Signature of opponent or representative

Place: Munich

Date: Aug. 09, 2017

Signature: Leo Polz

Name (block capitals): DR. L. POLZ
Hoffmann Eitle PartmbB, Association No. 151

In case of legal persons, signatory's position within company:

Opponent's reference: 198 441 u6/ne

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Zusatzblatt / Supplementary Sheet

IX. A. Publications (cont.)

7 D8: Thomas et al., Molecular Immunology, Vol. 33, No. 17/18, 1389-1401, 1996

8 D9: US 2003/0202972 A1

9 D10: Wang et al., J. pharm. Sci., vol. 96, no. 1, p. 1-26, Jan. 2007

10 D11: US 6,171,586 B1

11 D12: Maloney et al., Blood, vol. 90, no.6, 1997: pp 2188-2195

12 ANNEX I: Commercial monoclonal antibodies 2006 (all modes of administration)

13 ANNEX II: Commercial monoclonal antibodies 2006 (i.v. administration)

DR. L. POLZ
Hoffmann Eitle PartmbB, Association No. 151

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101
09. Aug. 2017

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European Patent Office

80298 München

Munich, August 09, 2017

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MILANO
MADRID

Hoffmann Eitle's professionals include:
European Patent Attorneys
Patentanwälte | German Patent Attorneys
Rechtsanwälte | German Attorneys at Law
British Patent Attorneys (regulated by IPReg)
Italian Patent Attorneys
Spanish Patent Attorneys
Dutch Patent Attorneys
Belgian Patent Attorneys
European Trademark Attorneys
European Design Attorneys

Our Ref.:	**198 441 u6/ne** **Eculizumab Composition / ALEXION**
Opposition to:	**EP 2 359 834 B1 (11001632.6)**
Proprietor:	**Alexion Pharmaceuticals, Inc.** **Cheshire, CT 06410 (US)**
Opponent:	**Hoffmann Eitle** **Patent- und Rechtsanwälte PartmbB** **Arabellastr. 30** **81925 Munich (DE)**

NOTICE OF OPPOSITION

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In the name and on behalf of

Hoffmann Eitle
Patent- und Rechtsanwälte PartmbB
Arabellastr. 30
81925 Munich (DE)

(hereinafter also referred to as the "***Opponent***")

we hereby lodge an

OPPOSITION

pursuant to **Art. 99 EPC** to

EP 2 359 834 B1

assigned to

Alexion Pharmaceuticals, Inc.
Cheshire, CT 06410 (US)

(hereinafter also referred to as the "***Proprietor***")

entitled:

"Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement".

A debit order to our account in the amount of **€ 785** is enclosed in accordance with **Art. 99(1) EPC** for the payment of the opposition fee.

Our requests and the statement of the grounds of the opposition are set out below.

REQUESTS

On the grounds of **Art. 100(a) EPC** it is petitioned that:

(1) the opposed patent **EP 2 359 834 B1** be revoked, and
(2) the parties be summoned to attend oral proceedings in accordance with **Art. 116(1) EPC** in the event that the Opposition Division is not in a position to immediately grant the request under (1).

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REASONS

(1) The claimed teaching according to **EP 2 359 834 B1**, hereinafter also referred to as the "***opposed patent***", does not comply with **Art. 56 EPC**, i.e. it does not involve an inventive step.

(2) References to the specification of the opposed patent or to other patent documents will be made – where appropriate – by citing the relevant paragraphs using their corresponding [4-digit] section numbers.

1. CITED PRIOR ART

(3) The present opposition is based on the following documents:

A	**WO 2007/106585 A1** (application as filed)
B	**EP 2 359 834 B1** (opposed patent)
C	**US 60/783,070** (priority application)
D1	**US 2005/0191298 A1**
D2	**Hillmen et al.**, British Journal Haematol., vol.121, Suppl. 1, p.87, **2003**
D3	**Hillmen et al.**, N Engl. J Med **2004**; 350: 552-559
D4	**Harding,** Drugs of the Future, Prous Science, ES, vol.29, no.7, 673-676, **2004**
D5	**US 2005/221382 A1**
D6	**Hill**, Clinical Advances in Hematology & Oncology, Millennium Medical Pub., New York, US, vol.3, no.11, 849-850, **2005**
D7	**US 6,355,245 B1**
D8	**Thomas et al.,** Molecular Immunology, Vol. 33, No. 17/18, 1389-1401, **1996**
D9	**US 2003/0202972 A1**
D10	**Wang et al., J. pharm. Sci., vol. 96, no. 1, p. 1-26, Jan. 2007**
D11	**US 6,171,586 B1**
D12	**Maloney et al.**, Blood, vol. 90, no.6, **1997**: pp 2188-2195

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ANNEX I Commercial monoclonal antibodies 2006 (all modes of administration)
ANNEX II Commercial monoclonal antibodies 2006 (i.v. administration)

(4) Documents **D1** to **D6** correspond to documents D1 to D6 as referenced in the **European Search Report** & **Opinion** dated July 12, 2011. Only **D1** and **D3** are refiled with this notice of opposition, as they will be dealt with in more detail below.

(5) Documents **D7 to D12**, are new documents introduced herewith for the first time into the proceedings.

2. THE OPPOSED PATENT

(6) The original application (**A**) was filed on March 15, 2007 and published as **WO 2007/106585 A1** on September 20, 2007. The opposed patent (**B**) was filed as divisional application **11001632.6** from parent application **07753249.7** and was granted on Nov. 9, 2016

(7) The opposed patent claims the priority of **US 60/783,070** (**C**) filed on March 15, 2006.

2.1 TECHNICAL OBJECTIVE OF THE OPPOSED PATENT

(8) The opposed patent relates the treatment of patients afflicted with ***paroxysmal nocturnal hemoglobinuria***, also referred to as "***PNH***" (cf. [0001]).

(9) "***Eculizumab***" is a humanized monoclonal antibody directed against the terminal complement protein C5. It was shown that eculizumab is able to reduce intravascular hemolysis when the antibody was administered intravenously to PNH patients (cf. [0003]). The results of a follow-up phase III study ("*TRIUMPH*") indicated that any treatment that decreases hemolysis in a patient would result in an improvement in the "*quality of life*" of said patient (cf. [0006]).

(10) The opposed patent seeks to provide a pharmaceutical composition for use in treating a patient afflicted with PNH.

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2.2 TECHNICAL SOLUTION AS CLAIMED

(11) As the technical solution to the above problem, the opposed patent was granted on the basis **claim 1**, which the sole claim of the patent. It reads:

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

2.2.1 Feature Analysis of Claim 1

(12) Accordingly, the pharmaceutical composition of Claim 1 is characterized by following technical features **1** to **7**:

1	*Medical use of the composition is for treating a patient afflicted with **PNH***
2	*the active ingredient of the composition is **eculizumab***
3	*the composition is a solution of a **300 mg eculizumab single-use dosage form***
4	*the **eculizumab concentration** in the composition is **10 mg / ml***
5	*the total **volume** of the composition is **30 ml***
6	*the solution is **sterile***
7	*the solution is **preservation-free***

2.2.2 Volume implicitly follows from of antibody concentration and antibody amount

(13) Notably, claim 1 stipulates that the total volume of the single-use dosage form is **30 ml** (feature 5). This feature, however, directly follows from features 3 and 4: Accordingly, a composition comprising 300 mg eculizumab at a concentration of 10 mg / ml would have a volume of 30 ml, as can be readily figured out by simple mathematical calculation.

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2.2.3 Mode of administration is not limited

(14) The pharmaceutical composition of claim 1 is not restricted to any particular mode of administration. According to paragraph [0058] of the description, administration of the antibodies will generally be performed by an intravascular route, e.g. via intravenous infusion by injection. However, "*other routes of administration may be used if desired*". These routes would include, for example, subcutaneous or intraperitoneal administration.

2.2.4 Method of manufacture is not limited

(15) It is further noted that opposed patent does not provide any specific method how to manufacture the pharmaceutical composition according to claim 1. The only information in this regard can be found in paragraph [0063] merely referring to prior art references, for example ***US 20030202972, US 20040091490, US 20050158316 WO 03/106644, WO 04/066957***, and ***WO 04/091658***.

(16) In this regard, reference is made to **US 2003/0202972**, provided herewith as **D9.** According to [0008] of **D9**, a lyophilized formulation can be reconstituted to generate a stable reconstituted formulation. For specific routes of administration, such as intravenous administration, concentrations of from about "***10 – 40 mg/ml protein in the reconstituted formulation***" are suggested (cf. [0107]).

(17) Hence, the antibody composition of opposed patent can be prepared and stored not only in liquid form, but also in lyophilized form, which is just reconstituted to a liquid solution with the features of claim 1 shortly before administration to the subject, for example, by intravenous infusion.

2.3 No Priority

(18) During the examination procedure, the proprietor relied, in particular, on page 5, lines 8-9, page 6, lines 21-23, and page 22, lines 12-14, of the application as filed (**A**) as basis for claim 1 of the granted patent (cf., for example, submission of January 13, 2016; p.2). These passages, however, are completely missing from the priority

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document **US 60/783,070 (C)**. In fact, there is no disclosure whatsoever on a specific eculizumab formulation in the priority application.

(19) It follows that the opposed patent does not validly claim the priority of **US 60/783,070**. The relevant date for defining the prior art in accordance with **Art. 54(2) EPC** is the filing date of the original application, i.e. **March 15, 2007**.

3. LACK OF INVENTIVE STEP (ARTICLES 100(A) AND 56 EPC)

(20) Claim 1 of the opposed patent does not involve an inventive step.

3.1 SKILLED PERSON IN THE ART AND COMMON GENERAL KNOWLEDGE

(21) The opposed patent relates to the use of an anti-C5 antibody, specifically the already known antibody eculizumab, for the treatment of PNH. Thus the skilled person was, in general, faced with the task of identifying appropriate dosage forms for proceeding forward with this treatment.

(22) The exercise of developing a suitable dosage form is the rudimentary basis to any clinical program. Such a program involves at least a formulation specialist, who is familiar with the manufacture of protein-containing, in particular antibody-containing compositions for administration to a human subject.

(23) This skilled practitioner may seek support, as necessary, from a clinician who is ultimately responsible for the clinical care of the patients (e.g. diagnosis, examination and determination treatment), or from a clinical pharmacologist, who provides dose and frequency recommendations on the basis of pharmacokinetics and pharmacodynamics.

(24) Said person skilled in the art, or team of experts, is possessed of average knowledge and ability. He or she is aware of what was common general knowledge in the art at the relevant date (cf., for example, **T 4/98, T 143/94** and **T 426/88**). Articles in technical journals can be representative of common general knowledge (cf. **T**

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595/90). This applies in particular to articles providing a **review** or **survey** of a topic (cf. **T 309/88**).

(25) The skilled person in the field of pharmaceutical drug development would have been aware of the common general knowledge, as reflected, for example, by **Wang et al. (2007)** (**D10**). This document was published in January 2007 and belongs to the state of art in accordance with **Art. 54(2) EPC**, since the opposed patent can only claim its actual filing date of March 15, 2007 (cf. point 2.3 above).

(26) **D10** reflects on pharmaceutical antibody formulations that have been approved by the health authorities before the filing date of the opposed patent. It is a **review article** and therefore belongs to the common general knowledge in the sense of **T 309/88**. As stated in the Abstract, by 2006 more than **23 monoclonal antibodies** have entered the US market. An overview on the properties of these commercial antibody products, such as administration route or antibody concentration, is given in **Table 1** of **D10**.

(27) It is readily presumed that a **formulation specialist would have consulted D10 for guidance when tasked with the manufacture of a therapeutical antibody formulation.**

(28) Document **D10** will be discussed in more detail below with respect to inventive step.

3.2 Closest Prior Art

(29) **US 2005/0101298** (**D1**) and **Hillmen et al. 2004** (**D3**) relate to the treatment of patients with infusions of eculizumab. As demonstrated in detail below, these documents relate to the same technical field (therapeutic use) as the opposed patent and would require the minimum of structural modifications to arrive at the teaching of claim 1 (cf. **T606/89**). Both documents therefore serve as alternative starting points, i.e. as closest prior art, for the assessment of inventive step according to the EPO's established "*problem-and-solution*" approach.

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3.2.1 Disclosure of US 2005/0101298 (D1)

(30) The disclosure of **D1** relates to a method of treating ***PNH*** in mammals (cf., for example, [0012], [0036]), in particular in human ***patients*** afflicted with this disease (cf. [0067]; Examples).

(31) The Examples report on the implementation of a clinical trial to evaluate the effects of an anti-C5 antibody on PNH and symptoms associated therewith (cf. [0081]). The anti-C5 antibody used was ***eculizumab*** (cf. [0082]; Figures).

(32) "***Eculizumab***" is the tradename for the anti-C5 antibody "***h5G1.1-mAb***" (cf. [0012], penultimate line; [0052]). According to section [0052] of **D1**, the **h5G1.1-mAb**, i.e. eculizumab, and methods for manufacturing it were already known from the prior art:

> *Particularly useful anti-C5-antibodies are* ***h5G1.1-mAb****, h5G1.1-scFv and other functional fragments of h5G1.1.* ***Methods for the preparation of h5G1.1-mAb****, h5G1.1-scFv and other functional fragments of h5G1.1* ***are described in U.S. Pat. No. 6,355,245*** *and "Inhibition of Complement Activity by Humanized Anti-C5 Antibody and Single Chains Fv",* ***Thomas et al.,*** *Molecular Immunology, Vol. 33, No. 17/18, pages 1389-1401,* ***1996, the disclosure of which are incorporated herein in their entirety by this reference****. The* ***antibody h5G1.1-mAb is currently undergoing clinical trials under the trade-name eculizumab****.*
> (cf. lower part of [0052]; emphasis added)

(33) **US 6,355,245** and **Thomas et al. 1996** are enclosed with this opposition brief as **D7** and **D8**, respectively. It will be appreciated, however, that according to the above quotation from [0052], both documents shall be regarded as incorporated in **D1** "*in their entirety*". That means that for applying the problem-and-solution approach the entire disclosure of **D1**, **D7** and **D8** **in combination** represent the "closest prior art"

(34) The mode of administration of eculizumab to a patient suggested by **D1** is, *inter alia*, "*via intravenous infusion*" (cf. [0060]) in a variety of "*unit dosage forms*" (cf. [0058]). The actual dosage regimen used in said clinical trial is described in [0082]:

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*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.*

(35) In summary, **D1** discloses the treatment of PNH patients with a pharmaceutical composition comprising eculizumab, said pharmaceutical composition being suitable for intravenous infusion.

3.2.2 Disclosure of Hillmen et al. 2004 (D3)

(36) **D3** is a scientific publication corresponding to the clinical study mentioned in the Examples of **D1**. It describes the details of the procedure and the results of the study in more detail. In particular, the clinical efficacy of eculizumab was tested in patients with PNH (cf. "*Background*"). Eleven transfusion-dependent patients with PNH received infusions of eculizumab (600mg) 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 (cf. "*Methods*").

Accordingly, **D3** basically comprises the same disclosure as **D1** and could likewise serve as the closest prior art. For the sake of convenience, the following assessment will be based on **D1** as the closest prior art.

3.3 OBJECTIVE TECHNICAL PROBLEM

(37) As outlined above, **D1** (or **D3**) disclose a *pharmaceutical composition for use in treating a patient afflicted with PNH* in the clinical study of the Examples. The active ingredient is eculizumab.

(38) Hence, features 1 and 2 of present claim 1 are disclosed in **D1**. Since the eculizumab comprising composition of **D1** is to be administered via intravenous infusion, it must be constituted as an aqueous solution.

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3.3.1 Feature comparison of Claim 1 with D1

(39) **D1** does not particularly specify how the composition is formulated, especially in terms of the absolute amount and concentration of eculizumab in the dosage form.

(40) As compared to **D1**, features 3 to 7 of granted claim 1 provide concrete characteristics of the pharmaceutical composition of the opposed patent. These are:

- the **total amount** of eculizumab in the single-use dosage form is **300 mg** (feature 3),
- the eculizumab **concentration** is **10 mg / ml** (feature 4),
- the **volume** is **30 ml** (feature 5), and
- the solution is **sterile** (feature 6), and
- the solution is **preservative-free** (feature 7).

3.3.2 No technical effect

(41) Features 1 to 7 in combination, in particular with regard to features 3 to 7, are described in sections [0017] and [0057] of the patent specification.

(42) No technical effects or advantages whatsoever contributed by said features 3 to 7, in terms of, for example, stability of the liquid formulation or shelf-life, are disclosed or merely suggested in the patent specification. No comparative experiments with other administration forms had been performed.

(43) Notably, the section "EXEMPLIFICATION" in the opposed patent, which describes the "TRUMPH" clinical study, only specifies the total amount of eculizumab (600 mg or 900 mg) administered to the patients via intravenous infusion (cf. [0074]), but is silent on the other features 3 to 7 of the pharmaceutical composition, such as eculizumab concentration in the solution etc.. Thus, it remains unclear whether the eculizumab formulation as claimed was actually used in the Examples of the opposed patent.

(44) In summary, when reading the specification as a whole, and in particular paragraphs [0063] to [0069] it becomes obvious that the **inventors considered the specific features of the eculizumab formulation rather conventional**. There is no suggestion

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whatsoever that there might be a surprising technical effect related to the claimed eculizumab composition, which is neither self-evident nor predictable.

3.3.3 Technical Problem as basis for the problem-and-solution approach

(45) The objective technical problem underlying the opposed patent is therefore to provide a pharmaceutical composition comprising eculizumab for use in the clinical studies described in any of **D1** or **D3**.

(46) Said composition shall be characterized in terms of the absolute amount and concentration of eculizumab, and be suitable for safe administration to human patients.

3.4 CLAIM 1 IS OBVIOUS OVER A COMBINATION OF D1 / D3 IN COMBINATION WITH US 6,171,586 (D11)

(47) In order to solve the objective technical problem of the opposed patent, the skilled person would have readily turned to **D11**.

3.4.1 Teaching of US 6,171,586 (D11)

(48) **D11** generally discloses a *stable aqueous **pharmaceutical formulation** comprising a therapeutically effective amount of an **antibody*** (cf. abstract; claim 1; col.2, l.25-33). In this regard, **D11** points to typical problems in connection with biological proteins like antibodies, such as degradation, chemical and physical instability; the most common protein degradation pathways being protein aggregation, deamination and oxidation (cf. col.1, l.15-38).

(49) The specification of **D11** provides abundant technical teaching how to overcome these problems, for example, by carefully controlling buffer composition, pH, and additives, such as surfactant and polyol. Accordingly, an antibody composition of **D11** is stable at a temperature of about 2 – 8°C for at least one year, and/or is stable at a temperature of about 30°C for at least one month, and/or is stable following freezing and thawing of the formulation (cf. col.2, l.25-34).

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(50) The general principles of this document are not limited to a specific antibody, but are rather applicable to antibodies in general. This becomes clear already from the formulation of the technical object underlying **D11**:

> *There is a need in the art for a **stable aqueous pharmaceutical formulation comprising an antibody**, such as an anti-CD18 or anti-CD20 antibody, which is **suitable for therapeutic use.***
> (cf. col.2, l.19-22; emphasis added)

(51) The antibodies for incorporation into the stable pharmaceutical composition of **D11** can be directed to a vast number of highly different protein antigens. A non-limiting list of such antigens is provided in column 10, lines 1 to 67 of the specification. The wording "*such as*" in the above quotation makes it clear that the specific anti-CD18 or anti-CD20 antibodies just represent exemplary embodiments of suitable antibodies. The skilled person would understand that the examples of **D11** dealing with anti CD18 and CD20 antibodies just serve to demonstrate the applicability of the invention of **D11**, but would not be construed as limiting the scope of the invention in terms of the antibody specificity (cf. col.24, l.22-26).

(52) Further, the antibodies of **D11** are not limited to specific classes or types of monoclonal antibodies, including antibody fragments, chimeric or "*humanized*" forms (cf. col.8, in particular l.54). Even if not specifically mentioned in the specification, the person skilled in the art would readily understand that also eculizumab could equally well be processed into a stable and safe pharmaceutical composition applying the methods of **D11**.

(53) **D11** also relates to containers holding said pharmaceutical formulation (cf. col.2, l.34-39). Suitable containers include, for example, bottles and vials formed from glass or plastic. Exemplary containers may 3 to 100 ml glass vials (cf. col.24, l.7-21).

(54) It therefore follows that the skilled person was clearly **motivated** to turn to **D11** for guidance when faced with the task of providing a pharmaceutical antibody formulation for use in the clinical study of **D1**. In doing so, the skilled person would have arrived at the pharmaceutical antibody composition according to claim 1 without any inventive effort, as outlined below:

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3.4.2 Total amount of 300 mg eculizumab in a single use-dosage form (feature 3)

(55) In the clinical study of **D1**, patients received two distinct doses of the active ingredient: First, **600 mg** doses of eculizumab were intravenously administered every week for four weeks. Then doses of **900 mg** were administered every other week.

(56) It is immediately obvious to the skilled person that the composition should be formulated as a **single-use dosage form comprising 300 mg eculizumab** (feature 3). This would allow conveniently using one and the same dosage form for both regimens, i.e. **two** single-use dosage forms for the 600 mg administration, and **three** single-use dosage forms for the 900 mg administration.

3.4.3 Eculizumab concentration of 10 mg / ml (feature 4)

(57) For the clinical study of **D1**...

> *.... each of 11 patients received a weekly **600 mg intravenous infusion** of anti-C5 antibody for approximately **thirty minutes**.*
> (cf. **D1**, [0082]; emphasis added)

(58) The skilled person was therefore set to the task to select a concentration of the antibody and a volume such that two single-use dosage forms of the pharmaceutical formulation each comprising 300 mg antibody can be intravenously administered within a period of thirty minutes.

(59) In order to implement the specific concentration of antibody / volume, the skilled person would have found further guidance in documents relating to antibody formulations for pharmaceutical applications, for example, **D11**.

(60) Accordingly, the antibody concentration in the formulations of **D11** is typically from about 0.1 to about 50 mg / ml (cf. claim 13). Preferred antibody concentrations are mentioned in column 22, lines 13 to 17 of the specification:

> *From about 0.1 mg / ml to about 50 mg / ml, preferably, from about 0.5 mg / ml to about 25 mg / ml, and **most preferably** from 2 mg / ml to*

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> *about **10 mg / ml** is an exemplary antibody concentration in the formulation (cf. col. 22, l.13-17).*
> (emphasis added)

(61) The above passage is quoted from the general description of **D11** and does not relate to any antibody in particular. It would therefore, *prima facie*, be applicable to any antibody. It should be particularly emphasized that the antibody concentration of "***10 mg / ml***" is **expressly** mentioned in **D11** as belonging to the **most preferred** concentration range.

(62) It was obvious to the skilled person with a reasonable expectation of success that the antibody concentrations mentioned in **D11** would be also applicable for a pharmaceutical eculizumab formulation. Feature 4 relating to the antibody concentration of 10 mg / ml is therefore an entirely obvious choice.

(63) Regarding the general applicability of **D11** with respect to different antibodies, and regarding the skilled person's expectation that the most preferred antibody concentrations suggested in **D11**, in particular 10 mg / ml, would have successfully worked also for eculizumab, see also point 3.5 below.

3.4.4 Total volume 30 ml (feature 5)

(64) As explained above, the volume of the composition implicitly, but nevertheless directly and unambiguously, follows from the absolute amount (300 mg; feature 3) and the concentration of the active ingredient in the solution (10 mg / ml; feature 4). Since both features 3 and 4 are obvious as demonstrated above, also feature 5 relating to the volume of 30 ml is obvious (300 mg / (10 mg / ml) = 30 ml).

3.4.5 Solution is sterile (feature 6)

(65) It goes without saying that formulations to be used for *in vivo* administration to a human subject must be sterile. This is common general knowledge and is, of course, appreciated by **D11**, too:

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> *The formulations to be used for in vivo administration* ***must*** *be* ***sterile****. This is readily accomplished by filtration through* ***sterile*** *filtration membranes, prior to, of following preparation of the formulation.*
> (cf. col. 23, l.27-30; emphasis added)

(66) Feature 6 does therefore not make any inventive contribution.

3.4.6 Solution is preservative-free (feature 7)

(67) According to the original application of the opposed patent, the pharmaceutical antibody compositions **may** or **may not** include preservatives (cf. **A**, for example, p.6, l.20, and p.24, l.23). Notably, even in the specification of the patent as granted it is still mentioned that preservatives can be included in the composition, leaving some doubts on the scope of granted claim 1:

> *Wetting agents, emulsifiers and lubricants, [.....] and perfuming agents,* ***preservatives*** *and anti-oxidants* ***can also be present*** *in the pharmaceutical compositions of the application.*
> (cf. [0064]; emphasis added)

(68) A similar disclosure can be found in paragraph [0058] of the opposed patent, stating that the "*formulations may contain pharmaceutically acceptable auxiliary substances as required, such as [....],* ***preservatives****, [....], and the like*".

(69) It is important to emphasize that no specific technical effect has been demonstrated in regard of the absence of preservatives. The limitation to "*preservative-free*" was therefore an obvious choice without any inventive contribution.

(70) In any event, **D11** expressly discloses the feature "*preservative-free*" as an alternative to the presence of preservatives, thereby clearly suggesting that preservatives are not necessary to obtain stable pharmaceutical antibody formulations:

> *In one embodiment, the formulation contains the above-identified agents (i.e. antibody, buffer, polyol and surfactant) and is* ***essentially free of one or more preservatives****, such as benzyl alcohol, phenol, m-cresol,*

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chlorobutanol and benzethonium C1.
(cf. col. 22, l.62-64; emphasis added).

(71) In conclusion, the teaching of claim 1 does not involve an inventive step.

3.5 CLAIMED ANTIBODY CONCENTRATION IS COMMON GENERAL KNOWLEDGE

(72) The common general knowledge, as reflected, for example, by **D10**, confirms the universal applicability of **D11** for the manufacture of various stable antibody formulations, in particular with respect to the concentration of the antibody of **10 mg / ml**.

3.5.1 Antibody concentrations of D11 are typical for commercial antibody formulations

(73) As mentioned in point 3.4.3 above, the broadest range for the antibody concentration suggested in **D11** is from **0.1 mg / ml to 50 mg / ml**, a preferred range being from **0.5 mg/ml to 25 mg/ml**, and the most preferred range being from **2 mg/ml to 10 mg/ml** (cf. col.22; l.13-17).

(74) Table 1 of **D10** lists **23 commercial therapeutical products** comprising authorized antibodies. The antibody concentration in the formulation for each product is given in the column headed "***Mab Conc***". One can reasonably presume that a skilled person - commissioned to prepare a therapeutical formulation for a new antibody - would have oriented himself on existing commercial antibody formulations, in particular with respect of such a mandatory parameter as the antibody concentration. All the more so because the number of approved antibody at the priority date was only 23, i.e. fairly manageable.

(75) In the table of **ANNEX I** submitted with this opposition, the antibodies of Table 1 of **D10** were grouped according the three concentration ranges of **D11**. That is: if the concentration of a specific antibody formulation fell within the range the broadest range of 0.1 to 50 mg / ml, the box in the corresponding column was checked (5th column of ANNEX I). If the concentration fell into the preferred range of 0.5 mg / ml – 25 mg / ml, the box in this column (6th col.) was checked, too, and so forth. In case

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the antibody concentration was exactly 10 mg / ml, the box in the last column (10 mg / ml) was checked.

(76) Since the antibody formulation of the present invention is not limited to any mode of administration, also routes other than intravenous administration were considered. Moreover, since the formulation according to claim 1 can also be reconstructed from a lyophilized powder formulation, such commercial products were likewise included.

(77) As one can take from column 5 of **ANNEX I**, 20 out of 23 therapeutic antibody formulations **(87%)** have a concentration within the range of 0.1 to 50 mg / ml. Still 18 of 23 antibody concentrations **(78%)** fall within the preferred range of 0.5 to 25 mg / ml, and 9 out of 23 antibody concentrations **(39%)** are within the most preferred range of **D11**, i.e. 2 to 10 mg / ml. Most remarkably, the number of commercial antibody formulations having a concentration of exactly **10 mg / ml** was still as high as **22%** (5 out of 23)!

(78) The individual antibody concentrations of the respective antibodies vary within the above ranges, but it is to be emphasized that 1**0 mg / ml is the most commonly used concentration** (5 formulations, versus 2 formulations with 0.5 mg / ml or 2 mg / ml, respectively).

(79) The clear and straightforward preference for an antibody concentration of 10 mg / ml becomes even more manifest if only antibody formulations for intravenous administration (18 antibody products) are considered. According to **ANNEX II**, 18 out of 18 therapeutic antibody formulations **(100%)** have a concentration within the range of 0.1 to 50 mg / ml. 17 of 18 antibody concentrations **(94%)** fall within the preferred range of 0.5 to 25 mg / ml, and 8 out of 18 antibody concentrations **(44%)** are within the most preferred range of 2 to 10 mg / ml. The number of antibody formulations having a concentration of exactly **10 mg/ml** is still **22%** (4 out of 18).

3.5.2 Preferred antibodies of D11 are commercially used at a concentration of 10 mg / ml

(80) As an antigen of special interest **D11** mentions the **CD20 surface antigen** which can be targeted for treating B cell lymphomas (cf. col.2, l.59-61). Several antibodies

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against CD20 were known at the filing date of **D11**, including the chimeric antibody termed "***C2B8***" (cf. col.2, lines 14-18 & reference to US 5,736,137 by Idec Pharmaceutical Corporation). Idec's C2B8 antibody is also known by the INN (international nonproprietary name) "***rituximab***", see for example **Maloney et al. 1997 (D12**; ct. title).

(81) The antibody product "**Rituxan**" comprising rituximab is listed among the 23 approved pharmaceutical antibody formulation of **D10**. According to Table 1, no 16, Rituxan comprises "**10 mg / ml**" of antibody in a solution of intravenous administration.

(82) Feature 4 would therefore belong to the common general knowledge.

3.5.3 Concentration of 10 mg / ml was obvious to try

(83) Thus, the skilled person would conclude from these data that the antibody concentrations suggested in **D11**, in particular 10 mg / ml would be generally applicable for many different antibodies. In fact, every 5th approved antibody that was commercialized before the priority date of the opposed patent, was successfully used at exactly the same concentration as claimed in the opposed patent, i.e. 10 mg/ml.

(84) It was therefore obvious for the skilled person to try out this concentration also for eculizumab with a reasonable expectation of success. The selection of 10 mg/ml according to feature 4 does not involve an inventive step.

4. CONCLUSION

(85) It clearly follows from the above that the arbitrary provision of a formulation form is not patentable merely because it potentially "enriches" the already existing repertoire of formulation forms. All of the features in combination as claimed in the opposed patent were obvious over **D1** (or **D3**) in combination with **D11,** or common general knowledge as reflected by **D10.**

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(86) Consequently, the opposed patent does not meet the requirements of the EPC. It has therefore to be revoked *in toto*.

Leo Polz

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Association number 151

Enclosures:

- Debit order in the amount of **€ 785.00** for the payment of the opposition fee
- Documents under **SECTION 1** above (except for D2, and D4 to D6).

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Exhibit no. D3
of the letter / report / expert opinion / plaint / defense
dated 09.08.2017
HOFFMANN · EITLE
Patent- und Rechtsanwälte
81925 München, Arabellastr. 30

ORIGINAL ARTICLE

Effect of Eculizumab on Hemolysis and Transfusion Requirements in Patients with Paroxysmal Nocturnal Hemoglobinuria

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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.

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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.

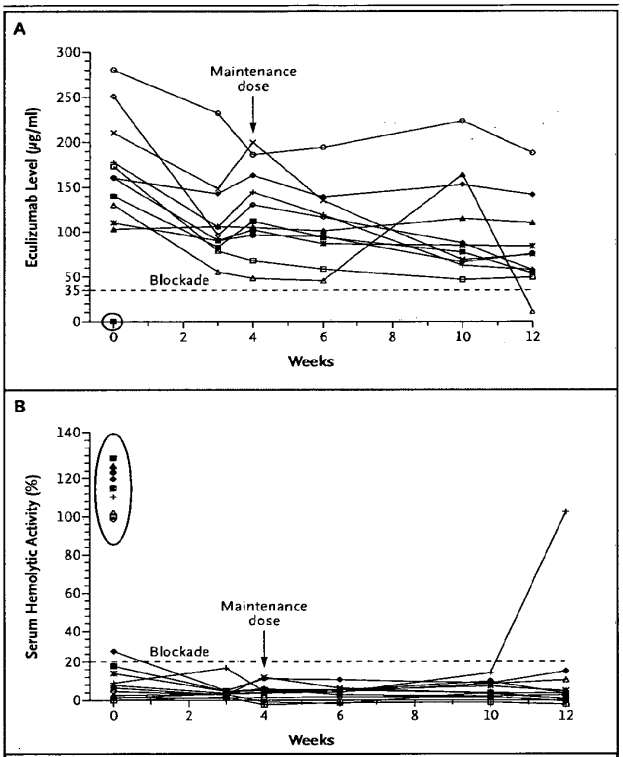


Figure 1. Pharmacokinetic and Pharmacodynamic Analysis of Eculizumab in 11 Patients with Paroxysmal Nocturnal Hemoglobinuria (PNH).

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).

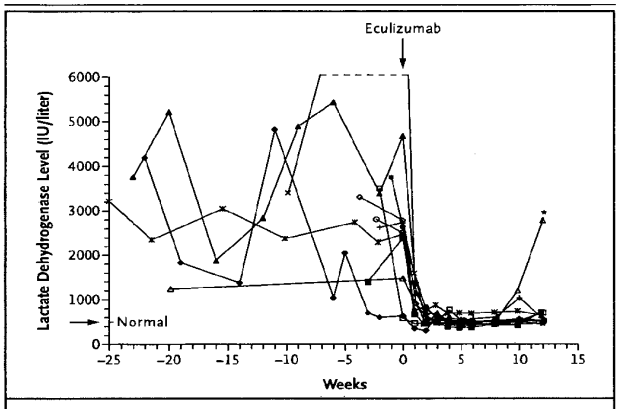


Figure 2. Analysis of Lactate Dehydrogenase Levels, a Biochemical Indicator of Hemolysis, in 11 Patients with Paroxysmal Nocturnal Hemoglobinuria up to 25 Weeks before and during 12 Weeks of Eculizumab Treatment.

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).

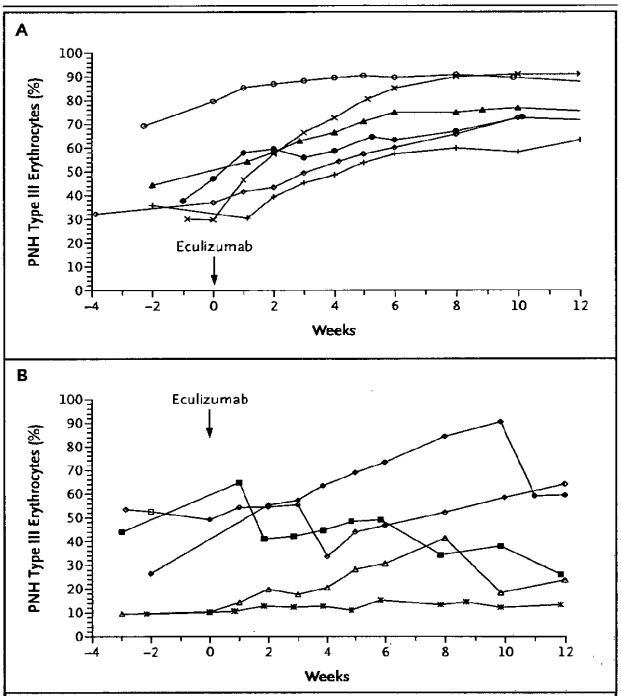


Figure 3. Changes in the Percentage of Paroxysmal Nocturnal Hemoglobinuria (PNH) Type III Erythrocytes during Eculizumab Treatment in Six Patients Who Received No Transfusions during Treatment (Panel A) and Five Patients Who Received at Least One Transfusion after Starting Treatment (Panel B).
The initial 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

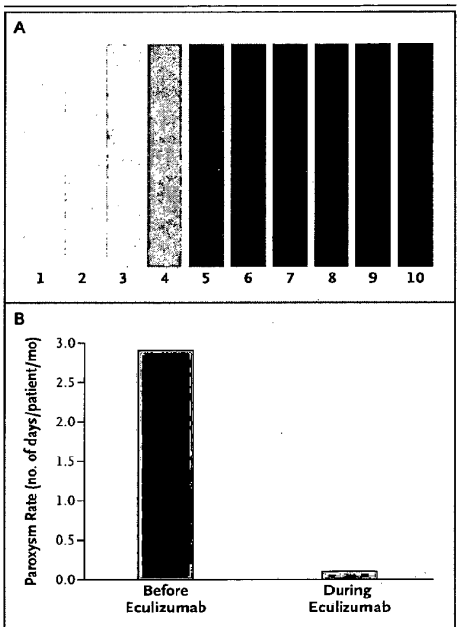


Figure 4. Incidence of Paroxysms during Eculizumab Treatment.

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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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.).

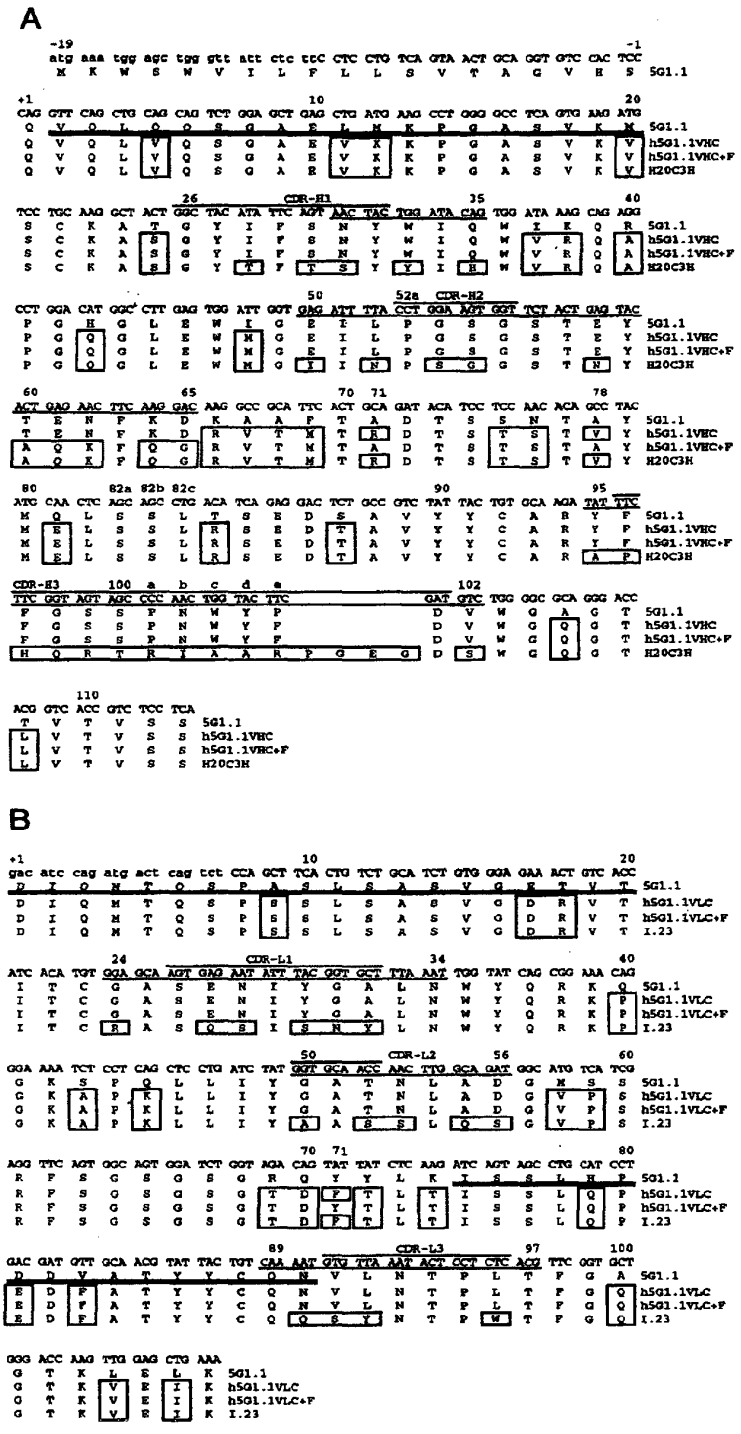
Construction of a humanized scFv

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

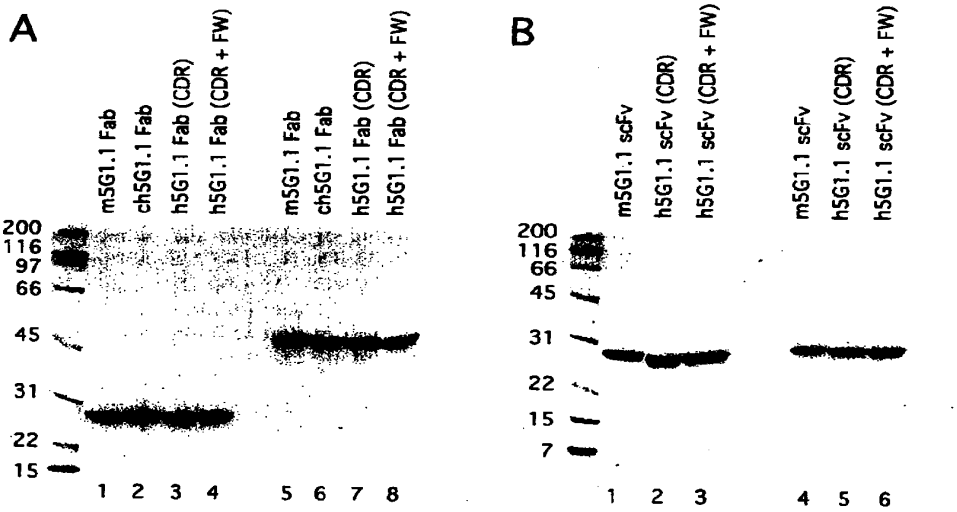


Fig. 2. SDS–PAGE analysis of purified recombinant proteins. (A) Fab fragments from the murine 5G1.1 mAb (m5G1.1 Fab) were produced by papain digestion of the m5G1.1 mAb followed by protein A chromatography to remove undigested antibody and Fc fragments. Recombinant chimeric Fab (ch5G1.1 Fab), CDR-grafted 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.

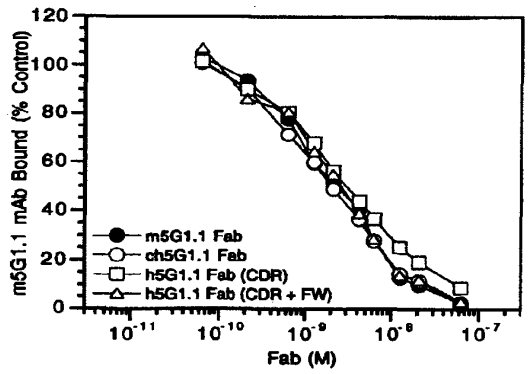


Fig. 3. Inhibition of 5G1.1 mAb binding to human C5 by recombinant Fab. 5G1.1 (0.2 nM) mAb plus the indicated concentrations of recombinant Fab were incubated together, for 2 hr at 37°C in microtiter plates coated with human C5. After washing, bound 5G1.1 mAb was detected using peroxidase-conjugated 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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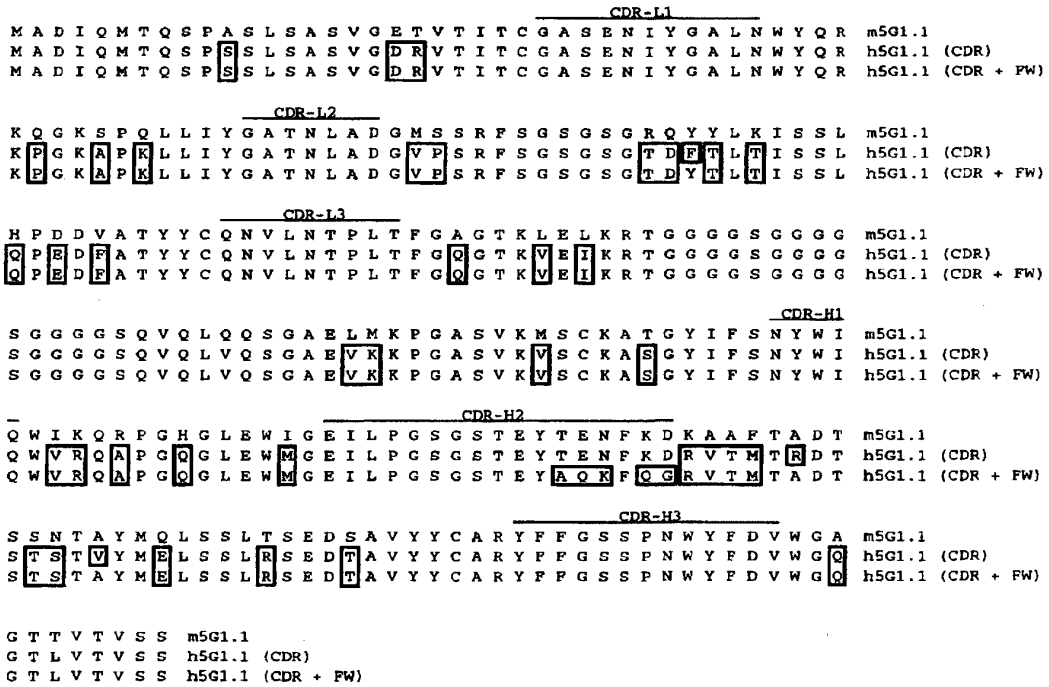


Fig. 4. Sequence of scFv molecules constructed using the 5G1.1 murine variable regions (m5G1.1) or humanized variable regions. The h5G1.1 (CDR) scFv contains the variable regions h5G1.1 VLC and h5G1.1 VHC, while the h5G1.1 scFv (CDR + FW) contains the variable regions h5G1.1 VLC+F and h5G1.1 VHC+F. The 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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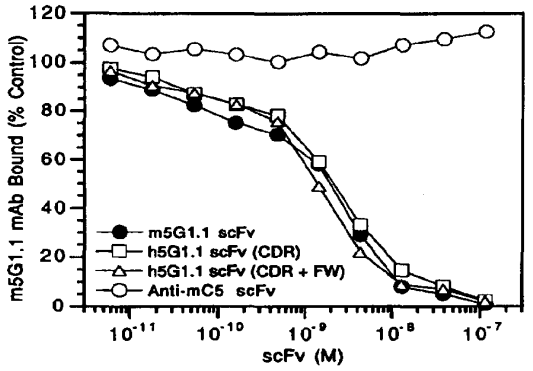


Fig. 5. Inhibition of 5G1.1 mAb binding to human C5 by recombinant scFv. 5G1.1 mAb (0.2 nM) plus the indicated concentrations of recombinant scFv were incubated together for 2 hr at 37°C, in microtiter plates coated directly with human C5. After washing, bound 5G1.1 mAb was detected using peroxidase-conjugated 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.

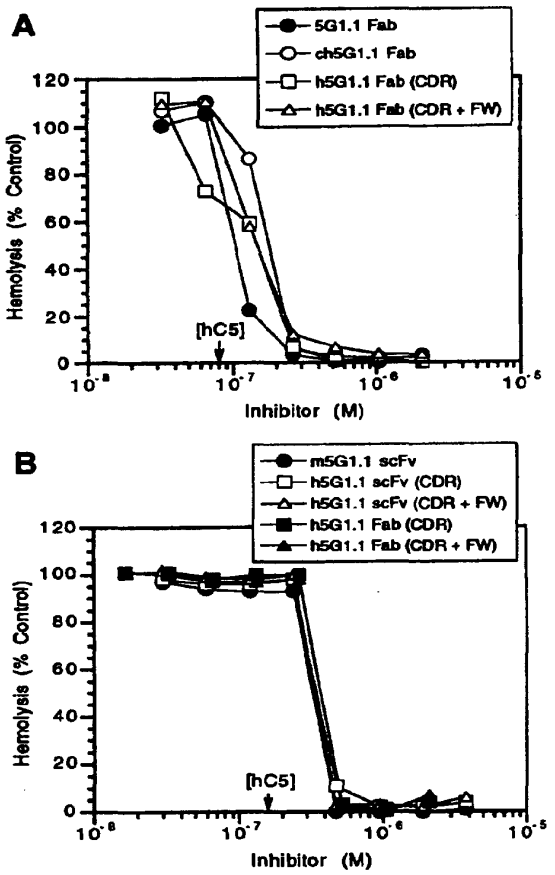


Fig. 6. Inhibition of chicken erythrocyte lysis by recombinant Fab and scFv. Twenty percent (A), or 40% (B), human serum in $GVBS^{++}$ was pre-incubated for 30 min at room temperature, with the indicated concentrations of competitors. Chicken erythrocytes, precoated with rabbit anti-chicken erythrocyte 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

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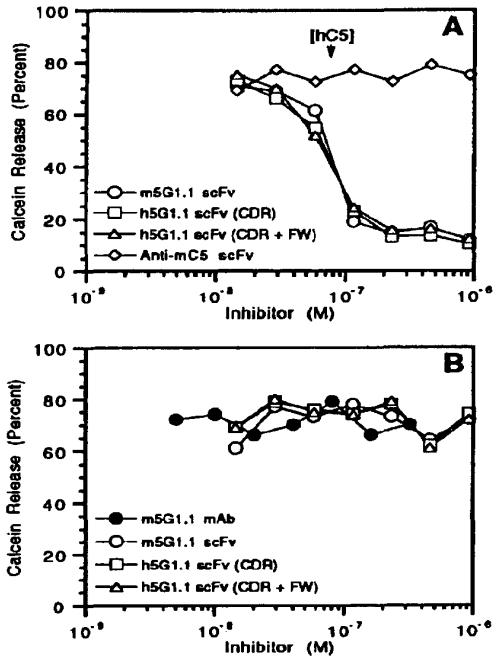


Fig. 7. Protection of porcine aortic endothelial cells (PAEC) from human serum complement by scFv. Calcein-labeled PAEC were incubated with HBSS containing 1% BSA and 0.85 mg/ml purified rabbit anti-PAEC polyclonal antibody for 30 min at 37°C. The cells were washed and incubated with HBSS containing 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.

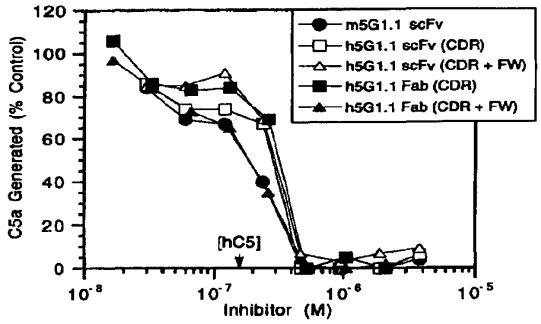


Fig. 8. Inhibition of C5a generation by recombinant Fab and scFv. Supernatants from chicken erythrocyte hemolytic assays performed using 40% human serum, as described in Fig. 5, were assayed for C5a content by ELISA. The C5a concentration obtained in the absence of inhibitor was defined as 100%. Data shown are from a representative experiment of several performed. The predicted hC5 concentration in 40% serum is indicated by the arrow.

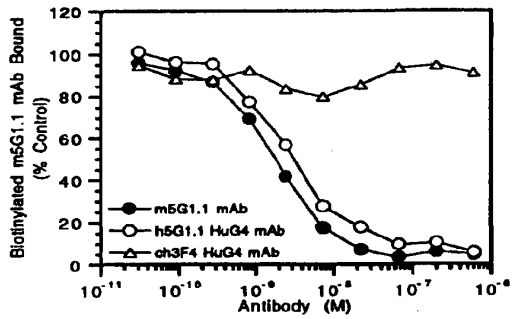


Fig. 9. Inhibition of 5G1.1 mAb binding to human C5 by recombinant humanized h5G1.1 HuG4 mAb. Biotinylated 5G1.1 mAb (0.2 nM), plus the indicated concentrations of unlabeled murine 5G1.1 mAb, recombinant h5G1.1 HuG4 mAb or a negative control recombinant HuG4 mAb (ch3F4 HuG4 mAb), were incubated together 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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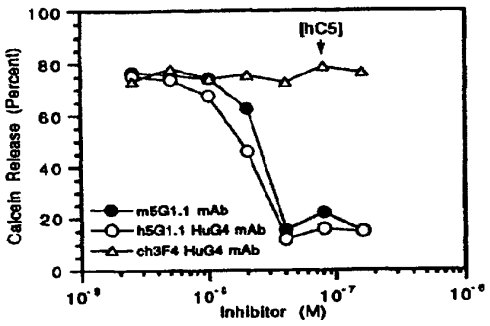


Fig. 10. Protection of porcine aortic endothelial cells from human serum complement by h5G1.1 HuG4 mAb. Calcein-labeled PAEC were incubated with HBSS containing 1% BSA and 0.85 mg/ml purified rabbit anti-PAEC polyclonal antibody for 30 min at 37°C. The cells were washed and incubated with HBSS containing 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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MINIREVIEW

Antibody Structure, Instability, and Formulation

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ABSTRACT: The number of therapeutic monoclonal antibody in development has increased tremendously over the last several years and this trend continues. At present there are more than 23 approved antibodies on the US market and an estimated 200 or more are in development. Although antibodies share certain structural similarities, development of commercially viable antibody pharmaceuticals has not been straightforward because of their unique and somewhat unpredictable solution behavior. This article reviews the structure and function of antibodies and the mechanisms of physical and chemical instabilities. Various aspects of formulation development have been examined to identify the critical attributes for the stabilization of antibodies. © 2006 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 96:1–26, 2007

Keywords: biotechnology; stabilization; protein formulation; protein aggregation; freeze drying/lyophilization

INTRODUCTION

Protein therapies are entering a new era with the influx of a significant number of antibody pharmaceuticals. Generally, protein drugs are effective at low concentrations with less side effects relative to small molecule drugs, even though, in rare cases, protein-induced antibody formation could be serious.[1] Therefore, this category of therapeutics is gaining tremendous momentum and widespread recognition both in small and large drug firms. Among protein drug therapies, antibodies play a major role in controlling many types of diseases such as cancer, infectious diseases, allergy, autoimmune diseases, and inflammation. Since the approval of the first monoclonal antibody (MAb) product -OKT-3 in 1986, more than 23 MAb drug products have entered the market (Tab. 1). The estimated number of antibodies and antibody derivatives constitute 20% of biopharmaceutical products currently in development (about 200).[2] The global therapeutic antibody market was predicted to reach $16.7 billion in 2008.[3]

There are several reasons for the increasing popularity of antibodies for commercial development. First, their action is specific, generally leading to fewer side effects. Second, antibodies may be conjugated to another therapeutic entity for efficient delivery of this entity to a target site, thus reducing potential side effects. For instance, Mylotarg is an approved chemotherapy agent composed of calicheamicin conjugated to humanized IgG4, which binds specifically to CD33 for the treatment of CD33-positive acute myeloid leukemia. Another example is the conjugation of immunotoxic barnase with the light chain of the anti-human ferritin monoclonal antibody F11 as potential targeting agents for cancer immunotherapy.[4] Third, antibodies may be conjugated to radioisotopes for specific diagnostic purposes. Examples include CEA-Scan for detection of colorectal cancer and ProstaScint for detection of prostate cancer. Lastly, technology advancement has made complete human MAb available, which are less immunogenic.

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Table 1. Commercial Monoclonal Antibody Products

#	Brand name	Molecule	MAb	Year	Company	Route	Indication	MAb Conc	Buffer	Excipients	Surfactant	pH
1	Avastin	Bevacizumab	Humanized IgG1, 149 kDa	2004	Genetech and BioOncology	IV infusion	Metastatic carcinoma of colon or rectum, binds VEGF	100 mg and 400 mg/vial (25 mg/mL) solution	5.8 mg/mL monobasic NaPhos H_2O; 1.2 mg/mL dibasic NaPhos anhydrous (4 mL, 16 mL fill in vial)	60 mg/mL α-Trehalose dihydrate (4 mL, 16 mL fill in vial)	0.4 mg/mL PS20 (4 mL, 16 mL fill in vial)	6.2
2	Bexxar	Tositumomab and I-131Tositumab	Murine IgG2λ	2003	Corixa and GSK	IV Infusion	CD20 positive follicular nonHodgkins lymphoma	Kit: 14 mg/mL MAb solution in 35 mg and 225 mg vials; 1.1 mg/mL *I131-MAb* solution	10 mM phosphate (MAb vial)	145 mM NaCl, 10 % w/v Maltose; *I131-MAb*: 5–6% Povidone, 1–2, 9–15 mg/mL Maltose, 0.9 mg/mL NaCl, 0.9–1.3 mg/mL Ascorbic acid		7.2
3	Campath	Alemtuzumab	Humanized, IgG1k, 150 kDa	2001	Ilex Oncology; Millenium and Berlex	IV infusion	B-cell chronic lymphocytic leukemia, CD52-antigen	30 mg/3 mL solution	3.5 mg/3 mL dibasic NaPhos, 0.6 mg/3 mL monobasic KPhos	24 mg/3 mL NaCl, 0.6 mg/3 mL KCl, 0.056 mg/3 mL Na2EDTA	0.3 mg/3 mL PS80	6.8–7.4
4	CEA-Scan (lyo)	Arcitumomab; Tc-99	Murine Fab, 50 kDa	1996	Immunomedics	IV injection or infusion	Imaging agent for colorectal cancer	1.25 mg/vial Lyophilized MAb. Reconstitute w 1 mL Saline w Tc99m	0.29 mg/vial Stannous chloride, potassium sodium tartrate tetrahydrate, NaAcetate.$3H_2O$, NaCl, glacial acetic acid, HCl	Sucrose		5.7
5	Erbitux	Cetuximab	Chimeric human/mouse IgG1k, 152 kDa	2004	ImClone and BMS	IV infusion	Treatment of EGFR-expressing colorectal carcinoma	100 mg MAb in 50 mL; 2 mg/mL solution	1.88 mg/mL DibasicNaPhos·$7H_2O$; 0.42 mg/mL MonobasicNaPhos·H_2O	8.48 mg/mL NaCl		7.0–7.4
6	Herceptin (lyo)	Trastuzumab	Humanized IgG1k	1998	Genetech	IV infusion	Metastatic breast cancer whose tumor overexpress HER2 protein	440 mg /vial, 21 mg/mL after reconstitution	9.9 mg/20 mL L-HistidineHCl, 6.4 mg/20 mL L-Histidine	400 mg/20 mL α-Trehalose Dihydrate	1.8 mg/20 mL PS20	6
7	Humira	Adalimumab	Human IgG1k, 148 kDa	2002	CAT and Abbott	SC	RA patients not responding to DMARDs. Blocks TNF-alpha	40 mg/0.8 mL solution (50 mg/mL)	0.69 mg/0.8 mL Monobasic NaPhos·$2H_2O$; 1.22 mg/0.8 mL DibasicNaPhos·$2H_2O$; 0.24 mg/0.8 mL NaCitrate, 1.04 mg/0.8 mL Citric acid·H_2O	4.93 mg/0.8 mL NaCl; 9.6 mg/0.8 mL Mannnitol	0.8 mg/0.8 mL PS80	5.2
8	Lucentis	Ranibizumab	Humanized IgG1k fragment	2006	Genentech	Intravitreal injection	Age-related macular degeneration (wet)	10 mg/mL solution	10 mM HistidineHCl	10% α-Trehalose-Dihydrate	0.01% PS20	5.5

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9	Mylotarg (lyo)	Gemtuzumab ozogamicin	Humanized IgG4k conjugated with calicheamicin	2000	Celltech and Wyeth	IV infusion	Humenized Ab linked to calicheamicin for treatment of CD33 positive acute myeloid leukemia	5 mg protein-equivalent lyophilized powder/20-mL vial	Monobasic and dibasic NaPhosphate	Dextran 40, Sucrose, NaCl		
10	OncoScint	Satumomab pendetide	Murine IgG1k conjugated to GYK-DTPA	1992	Cytogen	IV injection	Imaging agent for colorectal and ovarian cancer	0.5 mg conjugate/mL solution (2 mL per vial)	Phosphate buffer saline			6.0
11	Orthoclone OKT	Muromomab-CD3	Murine, IgG2a, 170 kDa	1986	Ortho Biotech	IV injection	Reversal of acute kidney transplant rejection (anti CD3-antigen)	1 mg/mL solution	2.25 mg/5 mL monobasic NaPhos, 9.0 mg/5 mL dibasic NaPhos	43 mg/5 mL NaCl	1 mg/mL PS80	7 ± 0.5
12	ProstaScint	Indium-111 capromab pendetide	Murine IgG1k-conjugated to GYK-DTPA	1996	Cytogen	IV injection	Imaging agent for prostate cancer	0.5 mg conjugate/mL solution (1 mL per vial)	Phosphate buffer saline			5–7
13	Raptiva (lyo)	Efalizumab	Humanized IgG1k	2003	Xoma and Genentech	SC	Chronic moderate to severe plaque psoriasis, binds to CD11a subunit of LFA-1	150 mg MAb/vial; 125 mg/ 1.25 mL (100 mg/mL) after reconstitution with 1.3 mL SWFI	6.8 mg/vial L-HistidineHCl·H_2O; 4.3 mg/vial L-IIistidine	123.2 mg/vial Sucrose	3 mg/vial PS20	6.2
14	Remicade (lyo)	Infliximab	Chimeric human/ murine MAb against TNFalpha (app. 30% murine, 70% corresponds to human IgG1 heavy chain and human kappa light chain constant regions)	1998	Centocor	IV infusion	RA and Crohn's disease (anti TNFalpha)	100 mg / 20-mL Vial, 10 mg/mL on reconstitution	2.2 mg/10 mL MonobasicNaPhos H_2O, 6.1 mg/10 mL DibasicNaPhos·$2H_2O$	500 mg/10 mL Sucrose	0.5 mg/10 mL PS80	7.2
15	ReoPro	Abciximab	Fab. Chimeric human-murine, 48 kDa	1994	Centocor / Lilly	IV injection and infusion	Reduction of acute blood clot related complications	2 mg/mL solution	0.01 M NaPhosphate	0.15 M NaCl	0.001% (0.01 mg/ mL) PS80	7.2
16	Rituxan	Rituximab	Chimeric mouse/ human IgG1k with murine light and heavy chain variable region (Fab domain), 145 kDa	1997	IDEC and Genentech	IV infusion	NonHodgkin's lymphoma. (anti CD20-antigen)	10 mg/mL solution	7.35 mg/mL NaCitrate·$2H_2O$	9 mg/mL NaCl	0.7 mg/mL PS80	6.5

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Table 1. *(Continued)*

#	Brand name	Molecule	MAb	Year	Company	Route	Indication	MAb Conc	Buffer	Excipients	Surfactant	pH
17	Simulect (lyo)	Basiliximab	Chimaric IgG1k, 144 kDa	1998	Novartis	IV injection and infusion	Prevention of acute kidney transplant rejection, IL-2 receptor antagonist	10 mg and 20 mg/ vial, 4 mg/mL on reconstitution	3.61 mg, 7.21 mg Monobasic KPhos; 0.50 mg, 0.99 mg Na_2HPO_4	0.8 mg, 1.61 mg NaCl; 10 mg, 20 mg Sucrose; 40 mg, 80 mg Mannitol; 20 mg 40 mg Glycine		
18	Synagis (lyo)	Palivizumab	Humanized IgG1k, CDR of murine MAb 1129, 148 kDa	1998	MedImmune	IM injection	Prevent replication of the Respiratory syncytial virus (RSV)	50 mg and 100 mg / vial, 100 mg/mL on reconstitution	47 mM Histidine, 3.0 mM Glycine	5.6 % Mannitol		
19	Tysabri	Natalizumab	Humainzed IgG4k	2004	Biogen IDEC	IV Infusion	MS relapse	300 mg/15 mL solution	17.0 mg MonobasicNa Phos·H_2O, 7.24 mg diBasicNaPhos·$7H_2O$ for 15 mL	123 mg/15 mL NaCl	3.0 mg/15 mL PS80	6.1
20	Verluma	Nofetumomab	Murine Fab	1996	Boehringer Ingelheim and DuPont Merck	IV injection	Imaging agent for lung cancer	10 mg/mL solution	Phosphate buffer saline			?
21	Xolair (lyo)	Omalizumab	Humanized IgG1k, 149 kDa		Genentech w Novartis and Tanox	SC	Asthma, inhibits binding of IgE to IgE receptor FCeRI	202.5 mg/vial, Deliver 150 mg/ 1.2 mL on reconstitution with 1.4 mL SWFI	2.8 mg LHistidineHCl·H_2O; 1.8 mg LHistidine	145.5 mg Sucrose	0.5 mg PS20	
22	Zenapax	Daclizumab	Humanized IgG1, 144 kDa	1997	Roche	IV infusion	Prophylaxis of acute organ rejection in patients receiving renal transplants. Inhibits IL-2 binding to the Tac subunit of IL-2 receptor complex	25 mg/5 mL MAb Solution	3.6 mg/mL MonobasicNa Phos·H_2O; 11 mg/mL DibasicNaPhos·$7H_2O$	4.6 mg/mL NaCl	0.2 mg/mL PS80	6.9
23	Zevalin	Ibritumomab-Tiuxetan	Murine IgG1k-thiourea covalent linkage to Tiuxetan		IDEC	IV infusion	CD20 antigen. (Kit with Yterium-90 induces cellular damage by beta emission)	3.2 mg/2 mL solution		09% NaCl		7.1

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Development of commercially viable antibody pharmaceuticals has, however, not been straightforward. This is because the behavior of antibodies seems to vary, even though they have similar structures. In attempting to address some of the challenges in developing antibody therapeutics, Harris et al.[5] reviewed the commercial-scale formulation and characterization of therapeutic recombinant antibodies. In a different review, antibody production and purification have been discussed.[2] Nevertheless, the overall instability and stabilization of antibody drug candidates have not been carefully examined in the literature. This article, not meant to be exhaustive, intends to review the structure and functions of antibodies, discuss their instabilities, and summarize the methods for stabilizing/formulating antibodies.

ANTIBODY STRUCTURE

Antibodies (immunoglobulins) are roughly Y-shaped molecules or combination of such molecules (Fig. 1). Their structures are divided into two regions—the variable (V) region (top of the Y) defining antigen-binding properties and the constant (C) region (stem of the Y), interacting with effector cells and molecules. Immunoglobulins can be divided into five different classes –IgA, IgD, IgE, IgM, and IgG based on their C regions, respectively designated as α, δ, ε, μ, and γ (five main heavy-chain classes).[6] Most IgGs are monomers, but IgA and IgM are respectively, dimmers and pentamers linked by J chains. IgGs are the most abundant, widely used for therapeutic purposes, and their structures will be discussed as antibody examples in detail.

Primary Structure

The structure of IgGs have been thoroughly reviewed.[6] The features of the primary structure of antibodies include heavy and light chains, glycosylation, disulfide bond, and heterogeneity.

Heavy and Light Chains

IgGs contain two identical heavy (H, 50 kDa) and two identical light (L, 25 kDa) chains (Fig. 1). Therefore, the total molecular weight is approximately 150 kDa. There are several disulfide bonds linking the two heavy chains, linking the heavy and light chains, and residing inside the chains (also see next section). IgGs are further divided

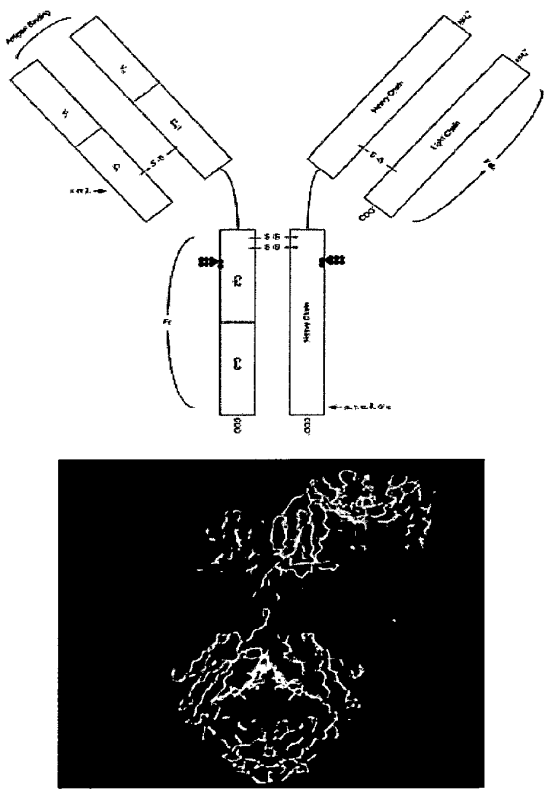


Figure 1. Linear (upper panel) and steric (lower panel) structures of immunoglobulins (IgG).

into several subclasses—IgG1, IgG2, IgG3, and IgG4 (in order of relative abundance in human plasma), with different heavy chains, named γ1, γ2, γ3, and γ4, respectively. The structural differences among these subtypes are the number and location of interchain disulfide bonds and the length of the hinge region. The light chains consist of two types—lambda (λ) and kappa (κ). In mice, the average of κ to λ ratio is 20:1, whereas it is 2:1 in humans.[6] The variable (V) regions of both chains cover approximately the first 110 amino acids, forming the antigen-binding (Fab) regions, whereas the remaining sequences are constant (C) regions, forming Fc (fragment crystallizable) regions for effector recognition and binding.[6] The N-terminal sequences of both the heavy and light chains vary greatly between different antibodies. It was suggested that the conserved sequences in human IgG1 antibodies

are approximately 95% and the remaining 5% is variable and creates their antigen-binding specificity.[5]

The V regions are further divided into three hypervariable sequences (HV1, HV2, and HV3) on both H and L chains. In the light chains, these are roughly from residues 28 to 35, from 49 to 59, and from 92 to 103, respectively.[6] Other regions are the framework regions (FR1, FR2, FR3, and FR4). The HV regions are also called the complementarity determining regions (CDR1, CDR2, and CDR3). While the framework regions form the β-sheets, the HV sequences form three loops at the outer edge of the β barrel (also see Section 2.2).

Disulfide Bonds

Most IgGs have four interchain disulfide bonds—two connecting the two H chains at the hinge region and the other two connecting the two L chains to the H chains.[6] Exceptions do exist. Two disulfide bonds were found in IgG1 and IgG4 linking the two heavy chain in the hinge region but four in IgG2.[7] In IgG1 MAb, HC is linked to the LC between the fifth Cys (C217) of HC and C213 on the LC. In IgG2 and IgG4 MAbs, it is the third Cys of HC (C123) linking to the LC.[7] A disulfide bond between HC C128 and LC C214 was found for mouse catalytic monoclonal antibodies (IgG2a).[8]

IgGs have four intrachain disulfide bonds, residing in each domain of the H and L chains, stabilizing these domains. The intrachain disulfide bonds in V_H and V_L are required in functional antigen binding.[9] Native IgG MAbs should not have any free sulfhydryl groups.[7] However, detailed examination of the free sulfhydryl groups in recombinant MAbs (one IgG1, two IgG2, and one IgG4) suggests presence of a small portion of free sulfhydryl group (approximately 0.02 mol per mole of IgG2 or IgG4 MAb and 0.03 for IgG1.[7] In rare cases, a free cysteine is found. A nondisulfide-bonded Cys at residue 105 was found on the heavy chain of a mouse monoclonal antibody, OKT3 (IgG2a).[10]

Oligosaccharides

There is one oligosaccharide chain in IgGs.[6] This N-linked biantennary sugar chain resides mostly on the conserved Asn 297, which is buried between the C_H2 domains.[5,11] For example, the oligosaccharide resides on Asn-297 of the C_H2 domain of chimeric IgG1 and IgG3 molecules[12] but on Asn 299 in a monoclonal antibody, OKT3 (IgG2a).[10] The oligosaccharide, often microheterogeneous, is typically fucosylated in antibodies produced in CHO or myeloma cell lines[5] and may differ in other cell lines.[2,11] There are many factors that dictate the nature of the glycan microheterogenity on IgGs. These include cell line, the bioreactor conditions and the nature of the downstream processing. An additional oligosaccharide can be found in rare cases. A human IgG produced by a human-human-mouse heterohybridoma contains an additional oligosaccharide on Asn 75 in the variable region of its heavy chain.[13] In addition, O-linked carbohydrates could also exist in this antibody.

Proper glycosylation is critical for correct functioning of antibodies.[11] It was demonstrated that removal of the oligosaccharide in IgGs (IgG1 and IgG3) made them ineffective in binding to C1q, in binding to the human FcγRI and activating C; and generally more sensitive to most proteases than their corresponding wild-type IgGs (one exception).[12] This is because the binding site on IgG for C1q, the first component of the complement cascade, is localized in the C_H2 domains.[11] Furthermore, the glycosylation can affect the antibody conformation.[12]

Oligosaccharides in other regions can also play a critical role. Removal of an oligosaccharide in a Fv region of the CBGA1 antibody resulted in a decreased antigen-binding activity in several ELISA systems.[13] In addition, this oligosaccharide might play critical role in reducing the antigenicity of the protein.[14]

The sugar composition of the oligosaccharide is also critical in antibody functions. It has been shown that a low fucose (Fuc) content in the complex-type oligosaccharide in a humanized chimeric IgG1 is responsible for a 50-fold higher antibody-dependent cellular cytotoxicity (ADCC) compared with a high Fuc counterpart.[15]

Heterogeneity

Purified antibodies are heterogeneous in structure. This is true for all monoclonal antibodies (MAbs) due to differences in glycosylation patterns, instability during production, and terminal processing.[5] For example, five charged isoforms were found in recombinant humanized monoclonal antibody HER2 as found by capillary isoelectric focusing (cIEF) and sodium dodecyl sulfate–capillary gel electrophoresis (SDS–CGE).[16] Six separate bands were focused under

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