Eculizumab prevents intravascular hemolysis in patients with paroxysmal nocturnal hemoglobinuria and unmasks low-level extravascular hemolysis occurring through C3 opsonization

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Funding: supported by an educational grant from Alexion Pharmaceuticals, Inc. to PH.

Acknowledgments: we would like to thank Gus Khursigara and Roseann Ventimiglia for critical review of the data and manuscript, Jason Chan for statistical analysis and Duncan S. Cole for information and supply of the FITC-conjugated anti-C3b antibody.

Manuscript received on February 9, 2009. Revised version arrived on September 23, 2009. Manuscript accepted on September 23, 2009.

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ABSTRACT

Background
Paroxysmal nocturnal hemoglobinuria is an acquired hemolytic anemia characterized by intravascular hemolysis which has been demonstrated to be effectively controlled with eculizumab. However, lactate dehydrogenase levels remain slightly elevated and haptoglobin levels remain low in some patients suggesting residual low-level hemolysis. This may be due to C3-mediated clearance of paroxysmal nocturnal hemoglobinuria red blood cells through the reticuloendothelial system.

Design and Methods
Thirty-nine samples from patients not treated with eculizumab and 31 samples from patients treated with eculizumab were obtained (for 17 of these 31 samples there were also samples taken prior to eculizumab treatment). Membrane bound complement was assessed by flow cytometry. Direct antiglobulin testing was carried out using two methods. Lactate dehydrogenase was assayed to assess the degree of hemolysis.

Results
Three of 39 patients (8%) with paroxysmal nocturnal hemoglobinuria not on eculizumab had a positive direct antiglobulin test, while the test was positive in 21 of 31 (68%) during eculizumab treatment. Of these 21 patients who had a positive direct antiglobulin test during eculizumab treatment, 17 had been tested prior to treatment; only one was positive. Flow cytometry using anti-C3 monoclonal antibodies was performed on the 21 direct antiglobulin test-positive, eculizumab-treated patients; the median proportion of C3-positive total red blood cells was 26%. Among the eculizumab-treated patients, 16 of the 21 (76.2%) with a positive direct antiglobulin test received at least one transfusion compared with one of ten (10.0%) of those with a negative test (P<0.01). Among the eculizumab-treated patients, the mean hemoglobin value for the 21 with a positive direct antiglobulin test was 9.6±0.3 g/dL, whereas that in the ten patients with a negative test was 11.0±0.4 g/dL (P=0.02).

Conclusions
These data demonstrate a previously masked mechanism of red cell clearance in paroxysmal nocturnal hemoglobinuria and suggests that blockade of complement at C5 allows C3 fragment accumulation on some paroxysmal nocturnal hemoglobinuria red cells, explaining the residual low-level hemolysis occurring in some eculizumab-treated patients.

Key words: paroxysmal nocturnal hemoglobinuria, eculizumab, complement, hemolysis, C3.

Citation: Hill A, Rother RP, Arnold L, Kelly R, Cullen MJ, Richards SJ, and Hillmen P. Eculizumab prevents intravascular hemolysis in patients with paroxysmal nocturnal hemoglobinuria and unmasks low-level extravascular hemolysis occurring through C3 opsonization. Haematologica. 2010; 95:567-573. doi:10.3324/haematol.2009.007229

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Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hemolytic disorder. The disease evolves from the clonal expansion of hematopoietic stem cells that have a somatic mutation in *PIG-A* resulting in the partial or complete loss of specific glycosylphosphatidylinositol (GPI)-linked proteins.[1,2] This lack of GPI expression results in the loss of the terminal complement inhibitor CD59 from the surface of hematopoietic cells, leaving red blood cells susceptible to complement-mediated intravascular hemolysis and unregulated activation of platelet and endothelial cells. The resulting chronic hemolysis in PNH leads to a syndrome of debilitating morbidities that includes severe anemia, disabling fatigue, thromboembolism, renal impairment, abdominal pain, dysphagia, hemoglobinuria and deteriorating quality of life.[2-4]

Eculizumab (h5G1.1-mAb, Soliris, Alexion Pharmaceuticals) is a monoclonal antibody designed to target the complement protein C5 and prevent its cleavage.[5] C5 is the point at which the three pathways of complement activation converge. Complement inhibition at this stage blocks the generation of the powerful anaphylotoxin C5a and the formation of the cell-lytic C5b-9 complex regardless of the complement activation stimuli. Importantly, targeting C5 also preserves the early complement components of C3-mediated activity critical for the clearance of micro-organisms and immune complexes.[6]

Eculizumab was evaluated in 195 patients with PNH in clinical studies.[2,7-9] By inhibiting terminal complement activation, eculizumab dramatically reduced intravascular hemolysis, as measured by a reduction in levels of lactate dehydrogenase (LDH), leading to improvements in anemia, fatigue, and quality of life as well as reductions in blood transfusions and thrombosis. Interestingly, while LDH was reduced from approximately ten times the upper limit of the normal range to near normal values with eculizumab treatment, levels remained slightly elevated in some patients. Additionally, undetectable haptoglobin, elevated bilirubin, and a persistent reticulocytosis in some patients suggested an on-going, low level of hemolysis in the midst of terminal complement inhibition.

Logue and Rosse demonstrated more bound C3 on PNH erythrocytes during complement activation.[10] We hypothesized that the on-going low-level hemolysis during eculizumab treatment in some patients could be occurring through the extravascular compartment due to C3-mediated opsonization of PNH red cells and subsequent clearance through the reticuloendothelial system. PNH cells also lack the proximal complement inhibitor CD55 (DAF), a glycoprotein that blocks complement activation upstream of C5 by dissociation of C3 convertases (C4b2a, C3bBb).[11-13] Deficiency of CD55 may, therefore, contribute to the generation and deposition of C3 on the PNH red cell surface. We set out to determine whether the low-level residual hemolysis observed in the presence of terminal complement blockade in patients with PNH could be due to C3-mediated clearance of the PNH red cell.

Design and Methods

Experiments were carried out on EDTA anti-coagulated peripheral blood samples obtained from patients with PNH both treated and not treated with eculizumab. This study research was approved by the local ethics board and written informed consent was obtained from all patients before samples were taken. We obtained 39 samples from patients not treated with eculizumab and 31 samples from patients treated with eculizumab. We were able to obtain samples prior to eculizumab therapy from 17 of the 31 eculizumab-treated patients. Positive and negative controls samples were generated from red blood cells obtained from normal healthy volunteers. Eculizumab was dosed at 600 mg every 7 days for 4 weeks, 900 mg 7 days later, and 900 mg every 14 days as a maintenance dose. Eculizumab was given by intravenous infusion over 30 min and was well tolerated.

Preparation of cellular controls

A positive complement-labeled red cell control was produced using serum from a patient with cold hemagglutinin disease (CHAD) which contains anti-I antibody. Complement was inactivated by incubating this serum at 56°C for 20 min. Ten microliters of a 1/100 dilution of whole blood were incubated with 10 μL neat CHAD serum and 10 μL C8d serum for 1 h at 4°C and then for 30 min at 37 °C (Figure 1). A negative control was prepared in parallel by omitting the CHAD serum. To test the effectiveness of the complement labeling procedure, control cells were washed twice and then incubated in a microtiter plate at 4°C for 20 min in the dark with 10 μL 1/100 dilution of a fluorescein isothiocyanate (FITC)-conjugated anti-C3b antibody (Department of Medical Biochemistry and Immunology, School of Medicine, Cardiff University, Cardiff, UK). The cells were

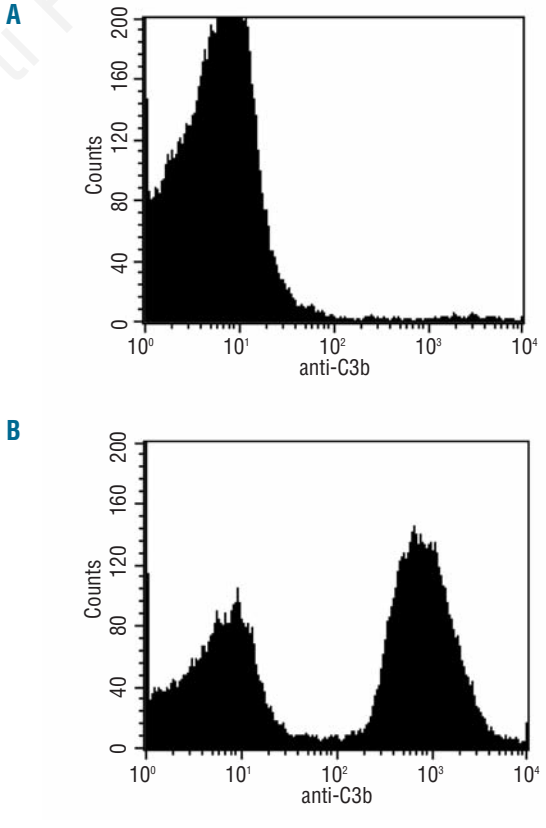

Figure 1. Surface levels of C3b fragment determined by flow cytometry. A fluorescent anti-C3b antibody was used against (A) washed whole blood from normal volunteers (negative control) and (B) washed whole blood from normal volunteers incubated with neat serum from a patient with cold hemagglutinin disease and neat C8d serum (positive control) (x axis = fluorescent intensity; log scale).

washed twice with 150 μL of FACSFlow (Becton Dickinson Biosciences, San Jose, CA, USA) and analyzed by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA) as detailed below.

Detection of membrane bound complement on paroxysmal nocturnal hemoglobinuria red cells by flow cytometry

Red cells from patients with PNH were tested for the presence of surface membrane-bound complement components using direct and indirect immunofluorescence procedures. For the detection of membrane bound C3b (C3 fragments), 10 μL of a monoclonal FITC-conjugated anti-C3b antibody (Department of Medical Biochemistry and Immunology, School of Medicine, Cardiff University, Cardiff, UK) were added to 10 μL of a 1/100 dilution of whole blood in a microtiter plate well. It has been demonstrated that monoclonal antibodies recognizing neoepitopes on C3b and other C3 fragments can be elicited by immunization of mice with native C3.[14] Kemp *et al.* went on to produce an antibody against surface-bound, but not fluid-phase, C3b by immunizing mice with denatured and reduced C3.[15] This antibody was the one used in the studies here. The cell antibody mixture was incubated at 4°C for 20 min in the dark and then washed twice with 150 μL volumes of FACSFlow (Becton Dickinson Biosciences, San Jose, CA, USA) to remove excess unbound antibody. The cell suspension was then resuspended in 300 μL of FACSFlow prior to flow cytometry acquisition. For two-color flow cytometry experiments, 5 μL of a phycoerythrin (PE)-conjugated anti-CD59 monoclonal antibody (Chemicon, Chandler's Ford, UK) were added. For the detection of surface membrane bound C3d, C1q, C5 and C8 components, anti-C3d, anti-C1q, anti-C5 and anti-C8 monoclonal biotinylated antibodies and a secondary PE-conjugated streptavidin reagent (Becton Dickinson Biosciences, San Jose, CA, USA) were used in an indirect procedure. Ten microliter volumes of a 1/100 dilution of red cells were incubated with 10 μL volumes of optimally titered dilutions of biotinylated antibodies. Cell antibody mixtures were incubated in microtiter plate wells for 15 - 30 min at 4°C. The red blood cells were then washed twice with 150 μL volumes of FACSFlow. Five microliters of the streptavidin PE-conjugate were then added to the red cells and incubated for a further 15 min at 4°C in the dark. The cells were then washed twice with 150 μL volumes of FACSFlow to remove excess unbound streptavidin conjugate. The cell suspension was then resuspended in 300 μL of FACSFlow prior to flow cytometry acquisition.

Flow cytometry acquisition and analysis

Red cells were processed through a FACSCalibur flow cytometer using CellQuest acquisition software (Becton Dickinson Biosciences, San Jose, CA, USA). Fifty thousand events were acquired with forward and side scatter detectors set in logarithmic mode and adjusted to allow visualization of red cells. Identification of the red cells was based on light scatter characteristics and the purity of the gating was confirmed using an anti-CD235a antibody (HMDS, Leeds, UK). Positivity for membrane-bound complement components was determined from single histogram analysis and defined as a mean fluorescent intensity of more than two standard deviations of the negative control (red cells from healthy volunteer donors). Positive and negative control red cells were included each time the procedure was undertaken.

Direct antiglobulin test

Direct antiglobulin tests (DAT) using monoclonal IgG and C3d were performed on the same samples. Two methods were utilized in all patients. The first was the DiaMed-ID Micro Typing System. A red cell suspension was made by adding 10 μL red cells to 1.0 mL of ID-Diluent. Fifty microliters of this red cell suspension were then added to each microtube of the DiaMed Card containing either monoclonal anti-IgG, -IgA, -IgM, -C3, -C3b, -C3c, -C3d or -C4. The card was then centrifuged for 10 minutes and the results read. A second method was also used to ensure consistency of results. The second method was the Ortho BioVue system (Ortho Clinical Diagnostics, High Wycombe, UK). This method utilizes column agglutination technology. Ten microliters of a 3% red cell suspension (made by adding 40 μL red cells to 1 mL saline) were added to chambers of the Ortho BioVue cassette containing anti-IgG, -C3b, and -C3d. The cassette was then centrifuged and the results read.

Assessment of hemolysis

Hemolysis was assessed by measuring levels of LDH at multiple time points during the study (normal range, 160 - 430 IU/L).

Statistical analyses

For comparison of eculizumab-treated patients receiving one or more transfusions who were either DAT-positive or DAT-negative, a Fisher's exact test was used, whereas a t-test was used for comparison of hemoglobin levels in eculizumab-treated patients who were either DAT-positive or DAT-negative.

Results

Direct antiglobulin test in patients with paroxysmal nocturnal hemoglobinuria treated or not treated with eculizumab

To investigate whether the residual hemolysis evident in some eculizumab-treated PNH patients may be explained by extravascular red cell clearance through C3-mediated opsonization, we performed a DAT on patients in the presence or absence of eculizumab. Using two different commercially available detection methods, three of 39 (8%) PNH patients not receiving eculizumab tested positive in the DAT (Figure 2). Two of the three patients with a positive DAT were positive for IgG and weakly positive for C3d while the additional DAT-positive patient was weakly positive for IgG only. By contrast, 21 out of 31 (68%) PNH patients receiving eculizumab were DAT-positive (Figure 2). Twenty of the 21 DAT-positive patients

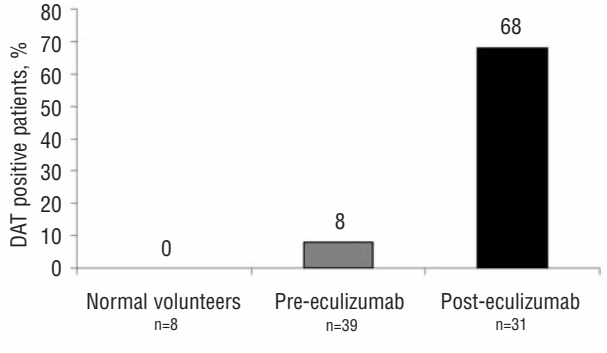

Figure 2. Direct antiglobulin test (DAT) in patients with PNH in the presence or absence of the complement inhibitor eculizumab. Proportions of DAT-positive individuals among normal volunteers, PNH patients not receiving eculizumab, and PNH patients receiving eculizumab are shown.

were positive for C3d and the one remaining was weakly positive for IgG as well as C3d. The median time of eculizumab treatment in the 21 patients who were DAT-positive was 11.5 months (range, 7-48 months) while the median time of eculizumab treatment in the ten patients who were DAT negative was 10.5 months (range, 9-47 months). All eight normal volunteers were DAT-negative. The median neutrophil PNH clone size in the DAT-negative patients was 90.2% (range, 33.8 to 99.9%) while that for the DAT-positive patients was 98.6% (range, 92.6 to 99.9%). There was also no difference in the population of type II PNH red cells in these groups (P=0.33). To confirm that DAT positivity was temporally related to eculizumab treatment, DAT results were analyzed for 17 DAT-positive patients during eculizumab treatment for whom DAT results prior to treatment were also available. Only one of these 17 (6%) patients was DAT-positive before eculizumab treatment.

Flow cytometric analysis of C3 deposition on red cells from patients with paroxysmal nocturnal hemoglobinuria treated or not with eculizumab

To confirm the results from the DAT showing that red cells from some patients with PNH treated with eculizumab accumulate C3 on their surface, flow cytometry with anti-CD59 and anti-C3b monoclonal antibodies was employed as a more sensitive measure. As expected, analysis of red cells from a normal individual failed to show any evidence of C3 fragment deposition (Figure 3, Panel A). Red cells from two patients with PNH who were not treated with eculizumab and were DAT negative showed low but detectable levels of C3 fragment deposition (Panels B and C) indicating that C3 deposition may occur on PNH cells at a level that is undetectable by DAT. By contrast, red cells from three patients who were receiving eculizumab therapy showed a distinct population of C3-positive cells (Panels D, E and F). Two-color flow

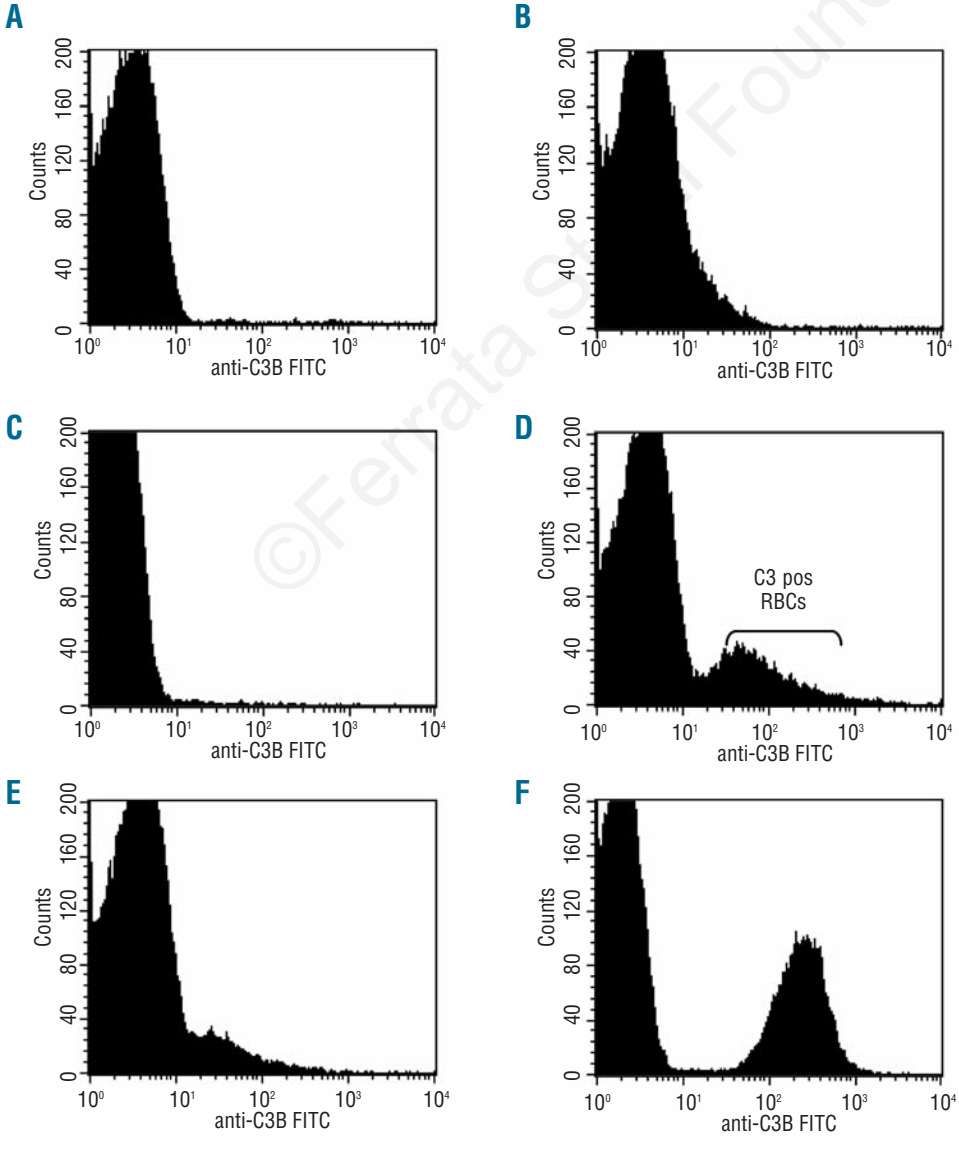

Figure 3. Flow cytometric analysis of C3b fragment deposition on red cells from PNH patients before or during eculizumab treatment. Levels of C3b on A) red cells from a normal volunteer; B) and C) red cells from a PNH patient not on eculizumab; D), E) and F) red cells from PNH patients on eculizumab.

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cytometry on 16 patients confirmed that the C3-positive population was CD59-deficient (Figure 4). No CD59-positive red cells had increased coating with C3.

By flow cytometry, the median and mean proportions of C3-coated red cells in 12 patients with PNH not receiving eculizumab treatment were 0% (range, 0% to 16%) and 2%, respectively. Of 21 DAT-positive eculizumab-treated patients tested, the median and mean proportions of total red cells with C3 expression were 26% (range, 0-53%) and 26%, respectively. By contrast, the median and mean proportions of C3-positive red cells in the ten DAT-negative eculizumab-treated patients were 5% (range, 1-14%) and 6%, respectively. There were no significant increases in C1q, C5 and C8 deposition in PNH patients on or off eculizumab.

Comparison of transfusion requirements, hemoglobin and lactate dehydrogenase levels in direct antiglobulin test-positive and -negative patients on eculizumab

Sixteen of the 21 (76.2%) DAT-positive patients on eculizumab received at least one transfusion during eculizumab therapy (median treatment duration of 11.5 months; range, 7 to 48 months) compared with one of ten (10.0%) DAT-negative patients (median treatment duration of 10.5 months; range, 9 to 47 months; $P<0.01$) (Figure 5). The reduction in transfusion requirements on eculizumab therapy was significant in both groups of patients when compared to pre-treatment requirements regardless of DAT positivity ($P<0.01$).

The mean hemoglobin value for the 21 DAT-positive patients on eculizumab was 9.6 ± 0.3 g/dL while the mean hemoglobin value for the ten DAT-negative eculizumab-treated patients was 11.0 ± 0.4 g/dL ($P=0.02$). Nine of the ten DAT-negative patients were also transfusion-independent. There was no significant difference in LDH levels in the two groups, further confirming the effectiveness of eculizumab in blocking the majority of the hemolysis. The median and mean LDH values for DAT-positive patients on eculizumab were 570 and 614 U/L, respectively, while the median and mean LDH values for eculizumab-treated DAT-negative patients were 588 and 562 U/L, respectively.

Discussion

The half-life of PNH red cells in patients with PNH may be as short as 3.5 days[16,17] compared with the normal red cell half-life of 60 days. The major cause of this markedly reduced half-life is most likely the chronic intravascular hemolysis occurring in patients with PNH. Eculizumab, a complement inhibitor recently approved for clinical use, has been shown in clinical trials to dramatically reduce hemolysis resulting in an increase in the half-life of PNH red blood cells.[2,7-9] Eculizumab treatment in PNH patients resulted in statistically significant reductions in thrombosis and transfusions as well as marked improvements in fatigue and quality of life.[8,9,18] Interestingly, in some eculizumab-treated patients, levels of LDH remained just above the upper limit of normal and haptoglobin remained undetectable. In addition, although hemoglobin levels improved in eculizumab-treated patients, in many cases they remained below the normal range.

By blocking the complement cascade with eculizumab, clinically evident intravascular hemolysis in PNH is abolished.[8] In this study, we tested for the presence of C3 fragments on the surface of PNH red cells as a possible mechanism for the residual hemolysis occurring in some eculizumab-treated patients. We observed increased levels of C3 fragments on the surface of PNH red cells in many patients who were receiving eculizumab, as also recently reported by Risitano *et al.*[19] The deposition of C3 fragments on PNH red blood cells in this setting could represent the revelation of a pathway of red cell clearance in PNH which is usually obscured by the rapidity of intravascular hemolysis in the absence of eculizumab therapy. Indeed, a small proportion of red cells from a patient with PNH not treated with eculizumab was shown by flow cytometric analysis to have C3 deposition that was unde-

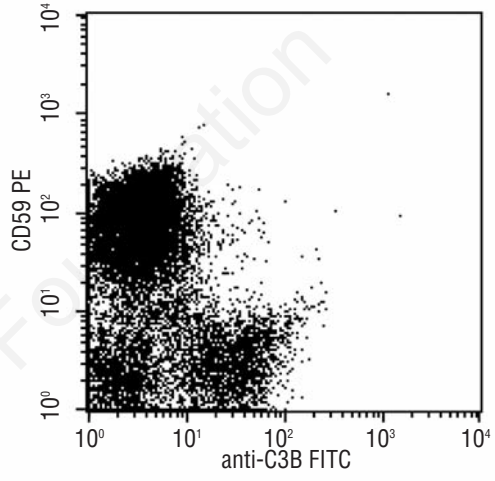

Figure 4. Two-color flow cytometric analysis of C3b fragment deposition on red cells from PNH patients during eculizumab treatment. A proportion of CD59-negative red cells are C3b-positive whereas the non-PNH (CD59-positive red cells) remain negative for C3b.

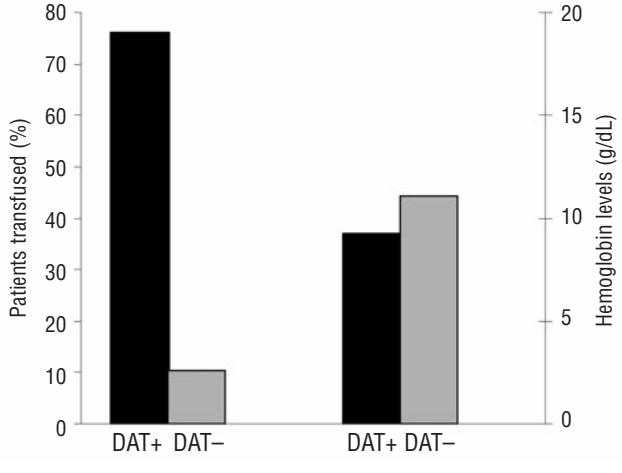

Figure 5. Transfusion requirement and hemoglobin values in DAT-positive or -negative PNH patients treated with eculizumab. Proportion of patients who received at least one transfusion and the level of hemoglobin in DAT-positive (DAT +; n=21) and DAT-negative (DAT -; n=10) PNH patients on eculizumab therapy.

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tectable by DAT. This is likely due to the lack of sensitivity of the DAT assay and demonstrates that low levels of C3 fragments may be present on PNH red cells. This deposition appears to be enhanced through blockade of terminal complement by eculizumab. Approximately 550-800 C3b molecules per red cell are required to activate the hepatic clearance mechanism through Kupffer cells.[20,21]

CD55 or DAF is a GPI-anchored protein that is also absent from the surface of the PNH red cell. CD55 is a complement regulatory protein that plays a key role in inhibiting the generation of C3b by regulating the formation of the C3 convertases and accelerating their dissociation.[22,23] The deficiency of CD55 in PNH red cells is likely to increase the generation of C3b and its fragments on the surface of these cells. However, individuals with the Inab phenotype (isolated CD55 deficiency) have no clinical evidence of extravascular hemolysis or increased levels of C3 fragments on their red cells.[24] Holguin and colleagues performed *in vitro* studies and blocked CD59 on Inab red cells; they found that the amount of C3 bound increased dramatically when the alternative pathway of complement was also activated by acidification of serum. They suggested that CD59 participates in the regulation of C3 convertase and may explain why PNH, but not Inab, red cells bind C3 *in vivo*. This is usually not clinically relevant as the cells are lysed through uncontrolled generation of the membrane attack complex. However, in the setting of eculizumab therapy, formation of the membrane attack complex is prevented and the importance of the CD55 deficiency in PNH revealed with extravascular clearance by C3 receptor-bearing cells of the reticuloendothelial system. This may explain, at least in part, the residual low level hemolysis occurring in some PNH patients on eculizumab therapy.

These studies have demonstrated increased C3 fragment deposition in some patients with PNH treated with eculizumab; however, the question as to why this does not occur in all patients (who are also CD55-deficient) remains unanswered. Possible explanations include differences in expression of CR1 (the transmembrane protein that controls the C3 convertase) or of red blood cell factor H binding sites, which could both contribute to the heterogeneity. However, both $CD59^{-}/C3^{+}$ and $CD59^{-}/C3^{-}$ populations are found in individual patients treated with eculizumab. Part of the problem may be the sensitivity of the analysis of C3 fragments on red cells or clearance of the affected cells. It is likely that the process occurs in all patients, as witnessed by the mildly raised LDH, reticulocytosis, raised bilirubin, anemia and low haptoglobin in PNH patients on eculizumab treatment.

An alternative mechanism of PNH red cell clearance was suggested by Jasinki *et al.*[25] Red blood cells from mice with PIG-A deficiency showed a reduced half-life that was complement-independent and likely due to increased clearance by fixed macrophages. This potential pathway, normally masked by the overwhelming complement-mediated intravascular hemolysis, may be manifest if the lytic activity of complement is blocked with eculizumab. The occurrence of this complement-independent pathway in patients with PNH has not yet been described.

The detection of red cell-bound immunoglobulins and complement by DAT remains the crucial serological assay in the diagnosis of autoimmune hemolytic anemia and is often used to rule out the diagnosis of PNH as anti-red cell antibodies are typically absent and the presence of C3 fragments on the surface of the PNH red cell is undetectable. Our data indicate that although most patients with PNH tested DAT-negative prior to eculizumab therapy, a subset of patients (8%) tested positive. Flow cytometry should, therefore, be considered to confirm or exclude PNH even in the setting of a positive DAT and hemolytic anemia when suspicion is high. Furthermore, many patients became strongly DAT positive following eculizumab therapy, which was not a result of the presence of anti-red cell antibodies but rather the deposition of C3 fragments.

In the previous double-blind, placebo-controlled TRIUMPH study, approximately 50% of eculizumab-treated patients became transfusion-independent while a 44% reduction in transfusions was observed in the remaining patients who did not achieve transfusion independence.[18] Although, it has not been definitively demonstrated that the requirement for residual transfusions in some eculizumab-treated PNH patients involves C3 fragment deposition on the red cell, such deposition was associated with lower hemoglobin levels and a higher likelihood of receiving at least one transfusion. However, there were DAT-positive patients receiving eculizumab who became transfusion-independent and patients who tested DAT-negative on eculizumab therapy who still required transfusions. These data strongly suggest that other factors are also involved in determining whether patients become transfusion-independent during eculizumab therapy. One factor is the underlying bone marrow insufficiency characteristic of PNH patients.

The novel mechanism of red cell clearance described herein may allow the development of strategies to further improve the response to eculizumab. Since C3-mediated clearance of red cells occurs through the reticuloendothelial system primarily in the spleen and liver, therapies such as steroids that affect this mechanism of red cell clearance may prove useful in this setting. Indeed, a recent study showed that splenectomy in a patient with PNH on eculizumab therapy resulted in a further reduction in transfusion requirements to a point that the patient became transfusion-independent.[26] However, concerns, particularly regarding thrombosis, do not make this an attractive option.

In conclusion, we describe a novel mechanism of PNH red cell clearance that has likely been unmasked due to the effective resolution of intravascular hemolysis by the terminal complement inhibitor eculizumab. Our data suggest that opsonization of PNH red cells by C3 fragments leads to extravascular clearance of these cells, and that this type of clearance may contribute to the low level of hemolysis and residual transfusion requirement observed in some patients on eculizumab therapy. Nonetheless, although this phenomenon may be recognized, it should not be taken as a reason to discontinue therapy as eculizumab therapy has proven to be an effective treatment for hemolysis and its untoward effects in patients with PNH. All patients participating in the current study have continued on therapy for up to 7 years.

Authorship and Disclosures

AH, RPR, SJR, and PH designed the research; AH performed the research; RPR, MJC, and SJR contributed vital new reagents or analytical tools; AH, LA, and RK collect-

ed data; AH, RPR, SJR, and PH analyzed and interpreted data; AH, and RPR performed statistical analyses; AH, RPR, SJR, and PH wrote the manuscript.

RPR is an employee of, and owns equity in Alexion Pharmaceuticals, Inc. He is also an inventor on patents assigned to Alexion but receives no royalties for these patents. SJR and PH have acted as consultants for Alexion Pharmaceuticals, Inc. in the past 2 years. PH has received research funding from Alexion Pharmaceuticals, Inc. AH, SJR and PH have received honoraria from Alexion Pharmaceuticals, Inc. AH, RK, SJR and PH have been members of an advisory board for Alexion Pharmaceuticals, Inc. No other potential conflicts of interests relevant to this article were reported.

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