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Complement Blockade with a C1 Esterase Inhibitor in Paroxysmal Nocturnal Hemoglobinuria

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Abstract

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare, clonal, hematopoietic stem cell disorder that manifests with a complement-mediated hemolytic anemia, bone marrow failure and a propensity for thrombosis. These patients experience both intra- and extravascular hemolysis in the context of underlying complement activation. Currently eculizumab effectively blocks the intravascular hemolysis PNH. There remains an unmet clinical need for a complement inhibitor with activity early in the complement cascade to block complement at the classical and alternative pathways. C1 esterase inhibitor (C1INH) is an endogenous human plasma protein that has broad inhibitory activity in the complement pathway through inhibition of the classical pathway by binding C1r and C1s and inhibits the mannose-binding lectin-associated serine proteases in the lectin pathway. In this study, we show that commercially available plasma derived C1INH prevents lysis induced by the alternative complement pathway, of PNH erythrocytes in human serum. Importantly, C1INH was able to block the accumulation of C3 degradation products on CD55 deficient erythrocytes from PNH patient on eculizumab therapy. This could suggest a role for inhibition of earlier phases of the complement cascade than that currently inhibited by eculizumab for incomplete or non-responders to that therapy.

Keywords

PNH; bone marrow failure; C3 blockade; complement; C1 esterase inhibitor

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Conflict of interest: Marc Uknis is Director of Clinical Research at ViroPharma.
JoAnne Saye is Director of Pre-Clinical Research at ViroPharma.

Background

Paroxysmal nocturnal hemoglobinuria (PNH) is a stem cell disorder that manifests with a complement-mediated hemolytic anemia, marrow failure and thrombosis [1-3]. Chronic hemolytic anemia in PNH is largely mediated by the alternative pathway of complement (APC). [2]PNH cells are deficient in glycosylphosphatidylinositol (GPI) anchored proteins including the complement regulatory proteins CD55 and CD59. [4]. CD55 regulates the formation and stability of the C3 and C5 convertases [1], whereas, CD59 blocks the formation of the membrane attack complex (MAC)[5] [2].

Eculizumab is an FDA-approved humanized monoclonal antibody that binds to C5. The drug decreases intravascular hemolysis, reduces thrombosis risk, and improves quality of life in PNH [6, 7] through inhibiting formation of the MAC [8]. Eculizumab compensates for the CD59 deficiency on PNH erythrocytes, but not the CD55 deficiency. Thus, PNH patients on eculizumab accumulate C3 fragments on their CD55 deficient red cells leading to extravascular hemolysis through the accumulation of opsonins that are recognized by the reticuloendothelial system [9]. Laboratory evidence of extravascular hemolysis in eculizumab-containing patients includes reticulocytosis, persistent anemia, and often direct Coombs test positive for C3 deposition. These patients may remain asymptomatic, but others have symptomatic anemia and remain dependent on transfusions. [10]. Thus, there is need for a complement inhibitor that reduces C3 accumulation on PNH erythrocytes.

C1 esterase inhibitor (C1INH) is an endogenous human plasma protein in the family of serine protease inhibitors (SERPINs) and it has broad inhibitory activity in the complement and coagulation pathways. C1INH inhibits the classical pathway of complement by binding C1r and C1s and inhibits the mannose-binding lectin-associated serine proteases in the lectin pathway.[11, 12] Thus, C1INH could be a therapeutic for diseases of the classical complement pathway and of the lectin pathway. In fact, plasma derived formulations of C1INH (Berinert, CSL Bering; Cetor, Sanquin, NL) have been evaluated for their clinical utility in pilot studies of sepsis, ischemia-reperfusion injury and capillary leak [13-16].

One proof of concept study investigating the role of C1INH for preventing hemolysis in PNH erythrocytes *ex vivo* showed that a commercially manufactured plasma derived C1INH (Baxter), further purified and concentrated by the investigators, inhibited PNH cell lysis by the APC and appeared to do so by inhibiting C3 and factor B binding to erythrocytes as well as inhibiting factor B and C3 cleavage[17]. A nanofiltered plasma derived C1INH (Cinryze®; ViroPharma) is FDA approved for routine prophylaxis against angioedema attacks in adolescent and adult patients with hereditary angioedema (HAE), a disease characterized by constitutional deficiency or dysfunction of endogenous C1 esterase inhibitor. Here we demonstrate that Cinryze (C1INH) inhibits C3 deposition fragments and the APC on PNH erythrocytes treated with eculizumab.

Material and Methods

Blood Samples

Peripheral blood of all patients was obtained by protocols approved by the Johns Hopkins institutional review board. PNH type III erythrocytes were stained with anti-CD55 defined as the percentage of CD55 deficient erythrocytes in whole blood and analyzed by flow cytometry using FlowJo software (www.treestar.com)[18, 19]. Patients were ages 18 years or older with a PNH type III erythrocyte proportion >5%. Clinical parameters for hemolysis were noted at the time of the sampling. To obtain eculizumab-containing serum, 20cc of peripheral blood was obtained from an atypical hemolytic uremic syndrome (aHUS) patient 30 minutes after receiving 1200mg of eculizumab, intravenously. The eculizumab-containing serum was stored at -80°C for all experiments to demonstrate C3 deposition.

C1 Esterase Inhibitor and Antibodies

Commercial vials of Cinryze® [plasma derived C1 esterase inhibitor (human)], or C1INH, were used for C1 inhibition assays *ex vivo*. Vials were reconstituted with distilled water (100U/ml) following the manufacturer's instructions. Serial dilutions of C1INH were prepared for dose response curves. PNH erythrocytes from the patients were incubated with either acidified human normal serum (aHNS, pH 6.4) or acidified eculizumab human serum (aEcuHS, pH 6.4) with or without C1INH. PNH erythrocytes from the patients with heat inactivated, acidified human serum (aNHS[H]) and acidified eculizumab-containing human serum (aEcuHS[H]) were used as baselines.

To identify the PNH erythrocyte population, the pellets were resuspended and stained with PE-conjugated anti-human CD55 antibody (clone: JS11, Cat. 311308, Biolegend), FITC-conjugated anti-human C3/C3b/iC3b antibody (clone: 7C12, Cedarlane Labs), and APC-conjugated anti-human CD235 (BD Biosciences). C3 deposition assays were performed by flow cytometry (BD LSRII BD Biosciences) using FlowJo software.

Hemolysis experiments for PNH Erythrocytes

PNH erythrocytes were centrifuged, the buffy coat was aspirated, and the cells were thoroughly washed 3 times with phosphate buffered saline (PBS). The PNH erythrocytes were then re-suspended in gelatin veronal buffer (GVB). The PNH erythrocytes were prepared to a hematocrit of 20% and kept at 4°C for no more than 2 weeks. [20]. Tests for the susceptibility of erythrocytes to APC-mediated lysis followed previously described methods.[20] Briefly, the PNH erythrocytes were washed with the GVB saline (pH 7.4), incubated at a final hematocrit of 20% and then diluted (1:3) with either acidified human sera (Type AB) or the eculizumab-containing serum from the aHUS patient. To estimate dose response *ex vivo*, C1INH of 0 U/mL, 3 U/mL, 6 U/mL, 9 U/mL and 12 U/ mL were added and incubated at 37°C for 1 hour. After 1 hour incubation, the erythrocytes were pelleted by centrifugation at 1500 rpm for 5 minutes and 50-100 μl of supernatant was collected and measured at 415 nm using iMark^{tm} Microplate reader (Bio-Rad). The percentage hemolysis was normalized and calculated based on 0% lysis (GVB only, pH 6.4) and 100% lysis (in distilled water).

C3 Inhibition

Flow cytometry was used to analyze deposition of C3 activation fragments on intact and lysed PNH erythrocytes (ghosts) from the patients. After 1 hour incubation with acidified human sera, GVB-EDTA was added to the erythrocytes to stop complement activity.

The pellets of ghosts and intact erythrocytes were collected by centrifugation at 1500 rpm for 5 minutes and washed three times with PBS (pH 7.4) and re-suspended in 0.5% BSA/PBS. The erythrocytes were stained with both FITC-conjugated anti-C3/C3b/iC3b antibody (1:10) and PE-conjugated anti-CD55 antibody (1:20) and C3 deposition was analyzed by flow cytometry as described above.

Results and Discussion

The clinical information for each patient is found in Table 1. These baseline data are presented to show persistent extravascular hemolysis despite long-term and ongoing inactivation of complement in patients receiving eculizumab therapy. Additionally, the data from Patient 6, who is *not* currently on eculizumab therapy, is shown.

C1 inhibition is concentration dependent

To investigate whether C1INH prevents complement-mediated hemolysis of PNH erythrocytes (from patients receiving eculizumab therapy) in a concentration dependent manner, the erythrocytes of patient 1 were treated with serial concentrations of C1INH (as described above in the methods) in the presence of both acidified normal serum (aNHS) and heat-inactivated acidified normal serum (aNHS[H]) . Figure 1A shows that, in the presence of the APC activation by aNHS compared to aNHS[H], the hemolysis of PNH erythrocytes is attenuated as the concentration of C1INH is increased in the sample. A concentration of C1INH at 6U/mL prevented hemolysis nearly to the level of aNHS[H] baseline. To further determine if the PNH erythrocyte percentage (amount of Type II versus Type III cells) affected the amount of lysis or the effect of C1INH, the PNH erythrocytes from patients 2-5 were co-incubated in aNHS and aNHS[H] with 0, 3, and 6Us/ml of C1INH. The results are presented in Figure 1B. This also demonstrates that the hemolysis is blocked in a concentration dependent fashion, regardless of the percentage of Type II or Type III erythrocytes present in the sample. We next tested whether C1INH would block hemolysis in PNH patient 6 who was *not* receiving therapy with eculizumab. Similarly to the patients on eculizumab therapy, C1INH prevented hemolysis *in vitro* in a dose dependent manner (Figure 1C).

CI Inhibition blocks APC-mediated deposition of C3 Fragments in Patients on Eculizumab Therapy

It has been demonstrated that patients with PNH on therapy with eculizumab develop extravascular hemolysis because the drug blocks terminal complement, thus allowing the CD55/CD59-deficient cells to become opsonized with activation and degradation products of C3 as a consequence of unrestricted activation of the APC [9, 21]. To investigate whether C1INH can protect cells from this C3 deposition, we exposed PNH erythrocytes to aEcuHS, aEcuHS[H], and aEcuHS + C1 (6U/mL) and assayed for deposition of C3 fragments. PNH

patients 2 & 6 are both CD55 deficient with only patient 2 currently receiving eculizumab therapy. C3 fragment deposition on the PNH erythrocytes was negligible in aEcuHS(H) (Figure 2, Top). The amount of C3 fragment deposition on the erythrocytes was increased (12.0 and 16.8%) in both subjects after incubation in aEcuHS (Figure 2, Middle). However, co-incubation of C1INH with the acidified, eculizumab-containing serum (aEcuHS + C1), C3 fragment deposition on the CD55 deficient erythrocytes was markedly attenuated (3.7% and 4.1%) (Figure 2, Lower).

Supplemental Figure 3S shows the percent hemolysis information for patients 2 and 6 using normal human serum (aNHS) as well as eculizumab-containing serum (aEcuHS). These data demonstrate near-complete hemolysis of the PNH red cells in activated normal human serum (aNHS) that is attenuated by heat inactivation, eculizumab-containing serum (aEcuHS) and additional C1INH .

We also measured C3 fragment deposition at baseline (T=0), after activation in eculizumab-containing serum and after the addition of C1INH in patients 2, 3, and 4. Supplemental Figure 4S confirms that C1INH inhibits the alternative pathway of complement by blocking the accumulation of C3 fragments on PNH erythrocytes.

In this study, we show that the commercially available plasma derived C1INH (Cinryze®) prevents PNH erythrocyte lysis induced by the alternative pathway of complement. Importantly, C1INH was able to block the accumulation of C3 degradation products on CD55 deficient erythrocytes from PNH patients on therapy with eculizumab. This activation of C3 to C3b on the surface of erythrocytes accounts for the immune-mediated, extravascular hemolysis that develops in patients on eculizumab therapy. This is clinically significant in patients treated with eculizumab who fail to achieve transfusion independence [10, 22, 23]. We and others have previously shown that breakthrough extravascular hemolysis can leave patients with only a partial response while on therapy with eculizumab [10, 22, 24]. Patients who do not respond to eculizumab therapy could theoretically respond to a C1 esterase inhibitor, either alone or in combination with C5 blockade. We hypothesize that a compound which targets the classical and the mannose complement pathways may be effective in APC-mediated hemolysis of PNH. The theory is that C1INH interacts with C3b to inhibit binding of factor B to C3b. At physiologic concentrations, this could down-regulate the activity of the alternative pathway. This could implicate a role for inhibition of earlier phases of the complement cascade than that currently inhibited by eculizumab for incomplete or non-responders to therapy with C5 blockade. A clinical trial to explore this hypothesis *in vivo* with patients who are suboptimal responders could be considered.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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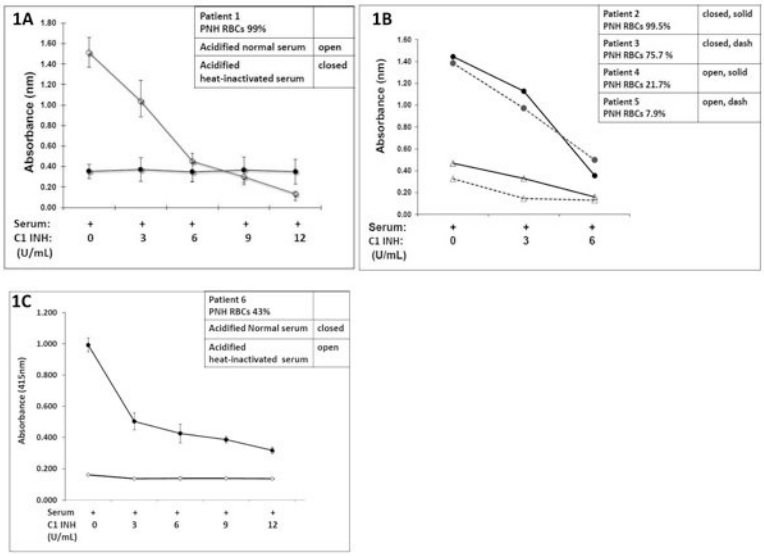

Figure 1. C1 Inhibition blocks APC-mediated Hemolysis in PNH Erythrocytes

A. The erythrocytes of patient 1 were incubated for 1 hour at 37° C with acidified (pH 6.4) normal human serum(aNHS) (1:3) and acidified heat-inactivated serum (aNHS[H])(1:3) containing increasing concentrations of C1INH (0-12 Units/mL). Hemolysis was measured using the concentration of supernatant hemoglobin (determined by spectrophotometry at 415 nm).

B. The erythrocytes of patients 2-5 (treated with eculizumab) were incubated as above with increasing concentrations of C1INH (0-6 Units/mL). Hemolysis was measured again by spectrophotometry at 415 nm.

C. The erythrocytes of patient 6 (not treated with eculizumab) were incubated as above at increasing concentrations of C1INH (0-12 Units/mL) and hemolysis measured.

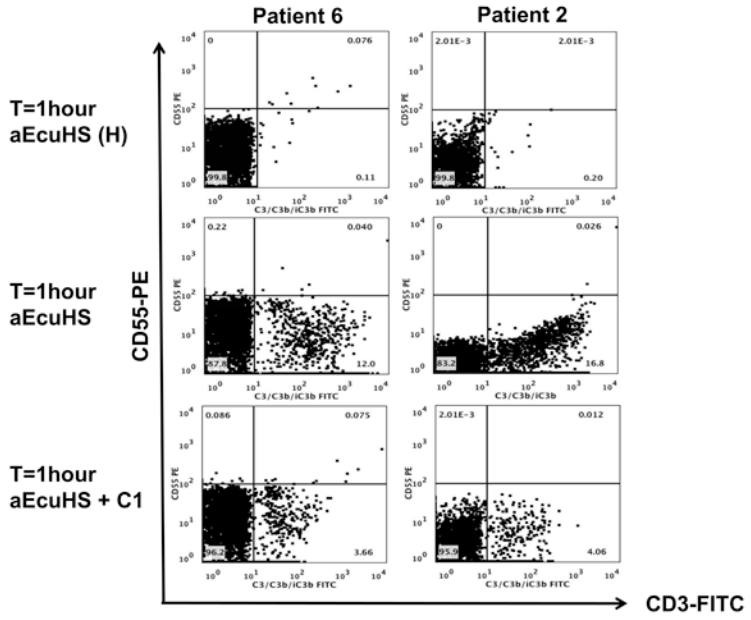

Figure 2. C1 Inhibition blocks APC-mediated C3 Fragment Deposition on PNH Erythrocytes

C3 fragment deposition was analyzed by flow cytometry LSRII (BD Biosciences) in Patient 2 (on eculizumab therapy) and Patient 6 (not on eculizumab therapy).

PNH erythrocytes were incubated at 37&degC for 1 hour with heat-inactivated eculizumab-containing serum, pH 6.4 (aEcuHS[H], Top), activated (pH 6.4) eculizumab-containing serum (aEcuHS, Middle), and activated (pH 6.4) eculizumab-containing serum plus C1INH at 6U/mL (aEcuHS + C1INH, Lower).

After incubation for 1 hour at 37°C, C3 deposition and CD55 were assayed by staining with FITC-conjugated anti-C3/C3b/iC3b antibody (C3-FITC) and PE-conjugated anti-CD55 antibody (CD55-PE).

C3 fragment deposition on the PNH erythrocytes was negligible in aEcuHS(H) (Top). The amount of C3 fragment deposition on the erythrocytes was increased (12.0 and 16.8%) in all subjects after incubation in aEcuHS (Middle). However, co-incubation of C1INH with the acidified, eculizumab-containing serum markedly attenuated (3.7% and 4.1%) C3 fragment deposition on the CD55 deficient erythrocytes (aEcuHS + C1) (Lower).

Table 1

Clinical Data on Patient Samples

Patient Number	Age Sex	Erythrocyte Clone Size* (%)	Granulocyte Clone Size* (%)	LDH (U/L)	Hemoglobin (g/dL)	Direct CoombsC3	Direct CoombsIgG	Absolute Reticulocyte Count (K/ cu mm)	Months on Eculizumab at q14days dosing	Days since last dose
1	25 F	Type II: 53 Type III: 46 TOTAL: 99	98	308	10.7	Positive	Negative	242	90	14
2	55 M	Type II: 0.8 Type III: 98.7 TOTAL: 99.5	98.7	318	10.6†	Positive	Negative	397	90	13
3	49 F	Type II: 4.7 Type III: 71 TOTAL: 75.7	96	301	10.1†	Positive	Negative	243	23	14
4	18 F	Type II: 8.7 Type III: 13 TOTAL: 21.7	92	326	13.2†	Positive	Negative	103	4	11
5	35 M	Type II: 2 Type III: 5.9 TOTAL: 7.9	100	312	15.9	Positive	Negative	145	55	14
6	37 M	Type II: 15 Type III: 29 TOTAL: 43	87	885	11.9	Negative	Negative	127	N/A	N/A

Clinical information from patients whose PNH erythrocytes were studied.
Patients 1-5 were on therapy with eculizumab for their disease at the time of study.
Patient 6 was not on eculizumab therapy for PNH.

*As measured in CLIA certified lab for clinical use

†Transfused in past year