

Monitoring and modeling treatment of atypical hemolytic uremic syndrome

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ABSTRACT

Atypical hemolytic uremic syndrome (aHUS), is mainly present in children, who have high risks of end-stage kidney disease (ESKD), post-transplant recurrence and death. aHUS is linked to defective regulation of the complement alternative pathway (AP), with a prominent cause being mutation/inhibition of the negative regulator complement factor H (CFH). CFH function can be restored *via* infusion of fresh frozen plasma (FFP), a treatment that was effective for several years in a patient heterozygous for a *cfh* mutation, before the patient progressed to ESKD. While on dialysis, FFP was replaced with eculizumab, which blocks C5 cleavage and thus halts progression of the terminal complement pathway. Patient plasma samples collected during FFP and eculizumab treatment phases were assessed for AP activity (*via* erythrocyte lysis assays) and for overall complement activity (*via* ELISA-based screen). Assay results indicated that FFP partially restored AP regulation, an observation supported by *in vitro* modeling of FFP treatment using purified CFH, while eculizumab completely blocked complement activity. The same approach was used to model *in vitro* a potential aHUS treatment approach based on blocking the AP effector properdin (complement factor P; CFP) with an anti-properdin antibody. These results provide insights into the efficacy of aHUS treatment and highlight the usefulness of *in vitro* assays in monitoring and predicting therapeutic responses and testing new treatment possibilities.

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1. Introduction

Atypical hemolytic uremic syndrome (aHUS) is a thrombotic microangiopathy (TMA) that mainly affects children; patients have high risks of end stage kidney disease (ESKD), post-transplant recurrence (<50%) and death (<25%) (Noris and Remuzzi, 2009). While understanding of aHUS pathology has advanced considerably in recent years, diagnostic and treatment standards are still lacking. Most aHUS cases are associated with defective regulation of the alternative pathway (AP) of complement system activation, arising from mutations and/or autoantibodies affecting complement AP regulators. For example >20% of aHUS patients have been found to carry mutant alleles of *cfh1* (1q32), which codes for complement factor H (CFH), the main inhibitor of complement AP activation in the fluid phase and on the surface of blood and vascular endothelial cells. Loss of CFH function results

in complement-mediated tissue damage to which the endothelium of the glomerular microvasculature is highly sensitive (Waters and Licht, 2011). To date >80 *cfh1* mutations have been described, most clustered in the C-terminal short consensus repeats (SCRs) (Pickering and Cook, 2008) where they disrupt CFH binding to surface membrane glucosaminoglycans and sialic acid residues that is essential for complement AP cell surface regulation by CFH (Heinen et al., 2007; Jokiranta et al., 2006; Manuelian et al., 2003).

For several years we have been following a patient heterozygous for an aHUS-associated *cfh1* mutation, c.3572C>T; p.Ser1191Leu (Olie et al., 2004; Rodriguez de Cordoba et al., 2004). Until recently she was successfully treated with fresh frozen plasma (FFP), which like all blood products incurs risks associated with infection, tolerance and resistance (Nathanson et al., 2006). Over time her aHUS ceased to respond to FFP, and while she remained FFP-dependent for the maintenance of a normal platelet count her progression to ESKD meant dialysis treatment had to be commenced. Recently, a new treatment option for aHUS appeared in the form of eculizumab, a humanized monoclonal antibody that blocks cleavage of C5 and thus prevents activation of the terminal complement cascade (Matis and Rollins, 1995; Parker et al., 2007; Thomas et al., 1996). Eculizumab has been successfully used in the treatment of paroxysmal nocturnal hemoglobinuria (PNH), which like aHUS is caused

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by defective complement regulation, and preliminary reports of eculizumab treatment in aHUS were promising (Hillmen et al., 2004, 2006). Our patient responded positively to eculizumab treatment, and we expect that in the near future she will receive a kidney transplant under eculizumab coverage (Weitz et al., 2011).

In the course of treating this patient we collected plasma samples during both the FFP and eculizumab treatment phases. We thus had the opportunity to assess the effects of both treatments on complement AP regulation *via* hemolysis assays, and on overall complement activity using an ELISA-based pathway-specific screen. Using the same assays we were also able to model the effects of aHUS treatment on patient plasma samples and test the potential of a novel strategy using an antibody to properdin (complement factor P; CFP) to specifically block complement AP activity. These results provide insights into the efficacy of aHUS treatment and highlight the usefulness of *in vitro* assays and models in monitoring and predicting therapeutic responses and guiding the development of more specific and efficient treatment strategies for aHUS patients.

2. Results

2.1. Evaluation of complement activity and regulation during treatment with plasma CFH replacement

A female aHUS patient heterozygous for the disease-associated *cfh1* mutation c.3572C>T; p.Ser1191Leu (Olie et al., 2004; Rodriguez de Cordoba et al., 2004) was treated for 11 years with fresh frozen plasma (FFP) infusions, typically 10–15 mL/kg/session every 14 days. This treatment was generally effective but the patient did experience periodic relapses/flare of acute disease when treatment intervals were increased in an effort to alleviate treatment burden. Eventually the patient's aHUS became refractory to FFP treatment and ESKD developed, at which point dialysis treatment (hemodialysis followed by peritoneal dialysis) was commenced. It was found necessary to continue plasma infusions in order to maintain normal platelet counts.

Plasma samples were collected during the responsive FFP treatment phase immediately before infusion (*i.e.* at functional CFH nadir) and after. Complement AP activity in plasma samples was assessed *via* a sheep erythrocyte hemolysis assay, where the ability of erythrocytes to bind human functional CFH inhibits complement AP-mediated cell surface complement activation leading to membrane attack complex (MAC) formation and lysis (*i.e.* loss of complement AP regulation is reflected by increased cell lysis). Plasma from the patient's mother (control 1) and a normal donor (control 2) showed low complement AP activity at all concentrations tested. Patient pre-infusion plasma showed a concentration-dependent increase in complement AP mediated hemolysis, indicative of a deficit in CFH function (Fig. 1A). All plasma samples showed equivalent ability to lyse rabbit erythrocytes, which cannot bind human CFH (data not shown) and thus serve as a control for competence of the terminal complement response.

2.2. Comparison of the efficacy of FFP and eculizumab treatments

The effect of individual FFP infusions on complement AP activity was assessed by sheep erythrocyte hemolysis assays of plasma samples collected during the FFP treatment phase at CFH nadir (above) and immediately following FFP infusion. Complement AP activity was clearly lower in post-infusion samples but remained elevated relative to normal donor plasma (Fig. 1B), indicating that FFP infusion had limited efficacy in restoring complement AP regulation, and that this efficacy waned between infusions.

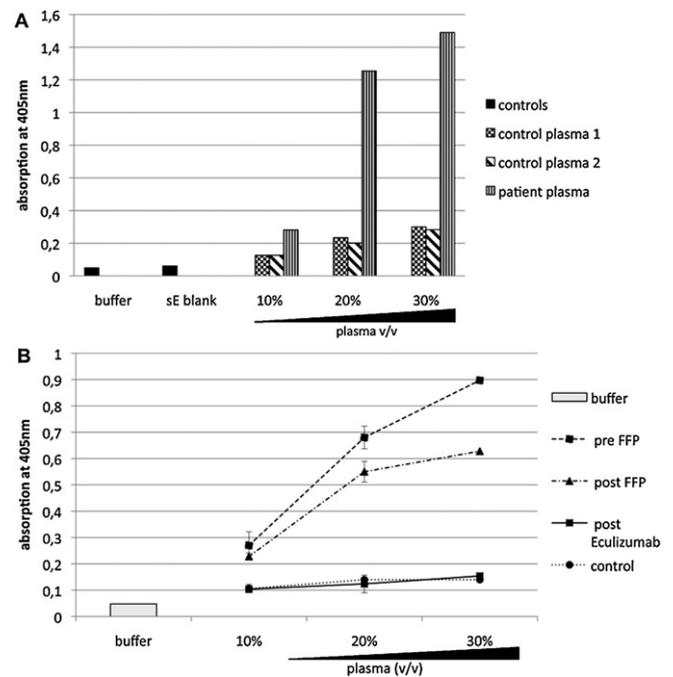


Fig. 1. Evaluation of complement AP dysregulation in aHUS. (A) Mutant CFH impairs complement AP regulation. Varying concentrations of plasma from the aHUS patient (containing both mutant CFH_{S1191L} and wild type CFH_{WT}), her mother (control plasma 1) and a healthy normal donor (control plasma 2) were tested for complement AP activity using the sE lysis assay. The low activity of maternal and normal donor plasma (indicated by hemoglobin absorbance at 405 nm) contrasts with the concentration-dependent increase in complement AP activity observed with patient plasma. (B) Eculizumab is superior to plasma infusion in down regulating complement AP activity. Patient plasma was diluted and tested for complement AP activity *via* hemolysis assay. Samples collected during the plasma treatment phase immediately before and after an FFP infusion (pre/post FFP) showed a decrease in complement AP activity post infusion, but activity remained high relative to normal donor plasma (control). Patient plasma collected after commencement of eculizumab treatment (post eculizumab) showed a marked decline to a normal low level of complement AP activity.

In contrast, plasma obtained after commencement of eculizumab treatment (induction: 900 mg/infusion/7 days \times 4 followed by 1200 mg/infusion/14 days) and cessation of plasma treatment showed a dramatic decline in complement AP activity to low normal levels, indicating high efficacy of eculizumab in blocking complement activation (Fig. 1B).

The effects of plasma and eculizumab treatment on total complement activity were further assessed using an ELISA-based screen that simultaneously measures activity of the complement AP, CP and MBL activation pathways in the same plasma sample (Seelen et al., 2005). In this assay, patient samples collected during the FFP treatment phase showed activity for all three activation pathways, with no difference evident between samples collected pre and post FFP infusion (Fig. 2A). Plasma samples collected after commencement of eculizumab treatment, however, showed total inactivity of all three activation pathways in samples taken before or after infusion (Fig. 2A).

2.3. *In vitro* modeling of aHUS FFP treatment with purified CFH

The effects of FFP treatment were simulated *in vitro* by repeating complement AP activity assays of aHUS patient pre-infusion plasma in the presence of purified functional CFH (pCFH). pCFH reduced complement AP activity in a concentration-dependent fashion until a normal level was reached at an added pCFH concentration of 30 μ g/mL (Fig. 2B). During a typical plasma treatment our patient received 10 mL/kg FFP infusion, which given the patient's

weight (50 kg) and the average concentration of CFH in normal plasma (500 $\mu\text{g}/\text{mL}$) was equivalent to adding 250 mg CFH to an estimated total plasma volume of 3.5 L (assuming 60 mL plasma/kg body weight), for a final added CFH concentration of approximately 71 $\mu\text{g}/\text{mL}$. Thus our *in vitro* modeling of aHUS plasma treatment indicates that a typical FFP infusion adds over twice the concentration of normal CFH required to restore full complement AP inhibition in patient plasma collected at the end of a treatment cycle. However, our assay results for post-FFP infusion plasma (Fig. 1B) indicate that this treatment does not immediately restore normal levels of complement AP inhibition.

2.4. Blocking of properdin (CFP) inhibits complement alternative pathway activation without affecting other modes of complement activation

Eculizumab comprehensively blocks complement activation (Fig. 2A). Since aHUS is specifically associated with loss of regulation of complement AP-mediated complement activation, the ideal aHUS treatment would address this deficit without paralyzing the entire complement response. In patients with aHUS-associated CFH mutation or inhibition (e.g. by autoantibodies), FFP infusion restores complement AP regulation by strengthening the inhibitory effects of CFH. A potential supplement and/or alternative to this therapeutic approach would be to target the complement AP

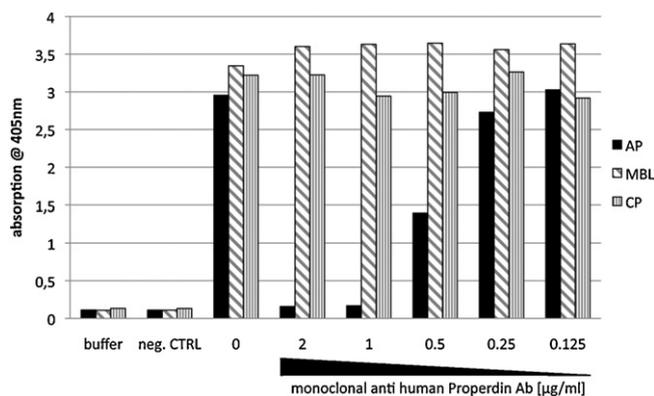


Fig. 3. Selective complement AP block via anti-properdin antibody. An ELISA-based complement screen was used to examine the effects of properdin inhibition on complement activity in normal donor serum. Monoclonal anti-properdin antibody specifically eliminated complement AP activity at 1–2 $\mu\text{g}/\text{mL}$ without affecting MBL and CP activity. Complement AP inhibition declined with antibody concentration and was lost below 0.25 $\mu\text{g}/\text{mL}$.

effector properdin, which is essential for the extension of the half-life of the complement AP C3 convertase (C3bBb) (Fearon and Austen, 1975; Medicus et al., 1976; Pangburn and Muller-Eberhard, 1986). Properdin deficiency has recently been shown to be beneficial in a complement AP-mediated mouse arthritis model (Kimura et al., 2010).

We examined the potential of this approach by adding anti-properdin blocking antibody to normal donor serum and measuring activity of specific complement pathways as described above. The anti-properdin antibody showed a concentration-dependent inhibition of complement AP activity, reaching a plateau of near-total blockade at a concentration of 1 $\mu\text{g}/\text{mL}$ (Fig. 3). Unlike eculizumab, the anti-properdin antibody specifically inhibited complement AP activity and did not affect CP or MBL activity (Fig. 3).

3. Discussion

Advances in the understanding of aHUS as a complement AP-mediated disease have contributed to recent progress in therapeutic management via treatments aimed at restoring functional complement AP control (e.g. via FFP treatment) or blocking the terminal complement cell-killing response (e.g. with the C5a blocker eculizumab). Plasma infusion/exchange is currently considered the gold standard for aHUS treatment, based on the rationale that plasma infusion replaces defective humoral complement regulators such as CFH (Fig. 2B). Although FFP or plasma exchange is the treatment of choice, little is known about its efficacy in terms of regaining complement AP control. Our *in vitro* measurements of the effects of FFP treatment showed that while complement AP regulation was improved it was not fully restored, since post-treatment patient plasma showed stronger hemolytic activity compared to controls (Fig. 1B: pre FFP/post FFP vs. control). A possible explanation for this could be insufficient activity and/or bioavailability of CFH present in FFP. It also remains possible that FFP treatment restores sufficient CFH function to provide effective complement AP regulation *in vivo* in concert with systemic factors including membrane-anchored complement regulators such as membrane cofactor protein (MCP; CD46), CR1 (CD35), CD55, CD59 and thrombomodulin (CD141) (Licht et al., 2009, 2005).

Our observations are consistent with clinical observations that the ability of FFP treatment to maintain complement control is limited. aHUS patients periodically experience relapses/acute episodes triggered by infection or injury (Caprioli et al., 2006), and some – like the patient studied here – eventually reach the point where plasma treatment can no longer halt disease progression.

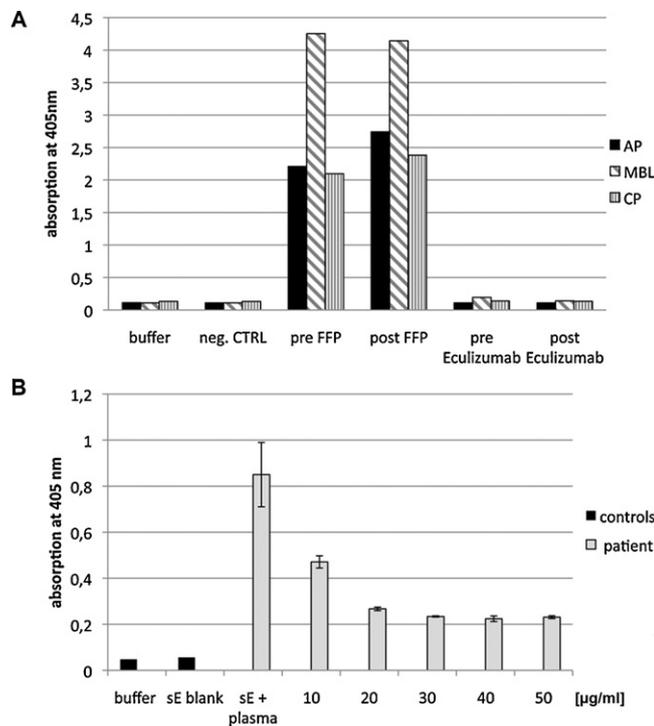


Fig. 2. Effects of FFP, eculizumab and CFH treatment *in vitro*. (A) Effects of FFP and eculizumab on complement activation pathways. Activities in patient plasma of three distinct complement activation pathways: complement alternative (AP), mannan-binding lectin (MBL) and classical pathway (CP), were assessed simultaneously using an ELISA-based complement screen. FFP infusion had no detectable effect on patient plasma activity (pre FFP vs. post FFP), while samples collected after commencement of biweekly eculizumab treatment showed no significant activity for all three activation pathways. Persistence of the eculizumab effect is indicated by the absence of complement activity in plasma collected both immediately before (pre eculizumab) and after (post eculizumab) a biweekly dose. (B) CFH restores complement AP regulation in aHUS patient plasma. Adding purified CFH to patient plasma samples restored complement AP regulation (i.e. reduced complement AP-mediated cell lysis) at concentrations >30 $\mu\text{g}/\text{mL}$ (controls: buffer only; sE blank = sE + buffer).

Such patients inevitably progress to ESKD and lifelong dependence on dialysis. Eculizumab offers a new treatment option in such cases (Al-Akash et al., 2011; Lapeyraque et al., 2011; Mache et al., 2009; Nurnberger et al., 2009), and our results demonstrate its effectiveness in shutting down complement activity in aHUS patient plasma tested after the onset of ESKD (Fig. 1B).

Eculizumab is clearly effective in blocking the cell-killing effects of complement *in vitro* and *in vivo*. There is, however, cause for caution in the treatment of conditions like aHUS. Its recent introduction means that there is little information available concerning the long-term effects of eculizumab treatment, especially in children. This makes it challenging to balance desired treatment effects – like stopping microvascular tissue damage – with unwanted consequences, such as immune system compromise. C5 activation and the terminal complement response play key roles in the detection and clearance of pathogens (e.g. encapsulated bacteria such as *Neisseria meningitidis* and *Streptococcus pneumoniae*) and in other aspects of host immune defense (Guo and Ward, 2005; Matis and Rollins, 1995; Parker et al., 2007; Thomas et al., 1996), thus C5 blockade could compromise the ability of patients to respond to infections and other insults. In the case of aHUS, it also seems clear that while total complement blockade may be effective in end stage disease or during acute flareups, effective restoration of complement AP regulation would likely be sufficient to prevent aHUS progression while preserving other aspects of complement-mediated innate immunity.

Thus while challenging in clinical practice, the optimal strategy for managing aHUS would be to therapeutically restore complement AP function as specifically and effectively as possible, and resort to total complement blockade with eculizumab or equivalents when – episodically or as a result of treatment failure – complement AP-directed treatment alone is insufficient to prevent complement-mediated tissue damage.

With this treatment ideal in mind, the results reported here point to some potential strategies for improvement in the management of aHUS patients. Our results using *in vitro* assays to assess complement activity in plasma samples demonstrate the potential utility of such technically modest tests in monitoring response to treatment in individual patients. Our results using purified CFH to model effects on complement activity *in vitro* indicate that in cases where aHUS is known to be linked to CFH functional defects or inhibition, the use of purified CFH may prove be more specific, effective and/or less susceptible to treatment failure than use of mixtures like FFP. Our *in vitro* modeling of the effects of properdin blockade point to another potential therapeutic target for regulation of complement AP activity in aHUS and other diseases, which could applied in concert with or as an alternative to existing treatments. Indeed in complement AP-related conditions like aHUS, specific properdin blockade may prove as effective in preventing complement-mediated damage as eculizumab, without affecting other aspects of the complement response.

4. Methods

4.1. Reagents

Sheep erythrocytes (sE), rabbit erythrocytes (rE) and anti-human properdin blocking monoclonal antibody (Quidel A233) were purchased from Cedarlane Canada (Burlington, ON, Canada). The Wieslab™ ELISA complement screen kit was from Alpco Diagnostics (Salem, NH, USA). Chemicals were from Sigma–Aldrich, Canada: HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid), N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid), EGTA (ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid), NaCl, MgCl₂.

4.2. Patient

This study was performed based on a protocol that was approved by the Research Ethics Board of The Hospital for Sick Children Toronto, ON, Canada. Written informed consent was obtained from the participant in accord with the Declaration of Helsinki.

The aHUS patient studied has been described in detail (De et al., 2010). Briefly, she is an adolescent heterozygous for a point mutation (c.3572C>T) in exon 22 of the human complement factor H gene (*cfh1*) resulting in an amino acid substitution (S1191L) in SCR 20 of CFH. This mutation affects the C-terminus of CFH which is involved with binding to cells, and is known to be associated with aHUS (De et al., 2010). Testing was negative for mutation of membrane cofactor protein (MCP; CD46), factor I (CFI), factor B (CFB) and *ADAMTS13*. Factor H related proteins 1 and 3 (CFHR1/3) were present; autoantibodies against CFH were not detected.

4.3. Plasma samples

Blood was obtained from the patient, her mother and other donors by venepuncture with citrate (3.2%) anticoagulation for plasma. Plasma and serum were prepared by high-speed centrifugation (2500 × g) followed by filtration (0.2 μm filter); aliquots were stored at –70 °C.

4.4. Plasma complement activity assay

Plasma samples were tested for complement activity by adding 15% (v/v) human plasma to 2×10^7 rabbit erythrocytes (rE) in complement AP buffer (20 mM HEPES, 10 mM EGTA, 144 mM NaCl, 7 mM MgCl₂, pH 7.4). Plasma and rE were incubated for 15 min at 37 °C. Cells were spun down and the supernatant was transferred to a 96 well microtiter plate, released hemoglobin was measured photometrically with an ELISA plate reader (Thermo Scientific) at 405 nm. A similar hemolysis assay using sheep erythrocytes was used to measure complement AP activity in plasma samples, which were also tested for activity of individual complement activation pathways (complement AP, CP and MBL) via the Wieslab™ ELISA-based complement screen, used according to the manufacturer's instructions. In this assay specific complement activation pathways are triggered via appropriate buffer conditions and activation surfaces, and complement activity is quantified via ELISA assay for membrane attack complex (MAC), C5b-9 (Seelen et al., 2005).

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