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Extravascular hemolysis and complement consumption in Paroxysmal Nocturnal Hemoglobinuria patients undergoing eculizumab treatment

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ABSTRACT

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hemolytic anemia characterized by complement-mediated intravascular hemolysis that is effectively treated with eculizumab. However, treatment responses are reported heterogeneous with some patients presenting residual hemolysis and requiring RBC transfusions. Recent reports have shown that both extravascular hemolysis and incomplete C5 blockade can explain these suboptimal hematological responses. Here we have tested our eculizumab-treated PNH patients (n = 12) for signs of hemolysis and assessed complement biomarkers. Patients were also genotyped for complement receptor 1 (CR1, CD35) and C5 polymorphisms and evaluated for free eculizumab in plasma. We report that 10 patients (83%) present parameters suggesting persistent hemolysis, although they did not require additional transfusions. Seven of them (58%) become direct Coombs-test positive as a consequence of treatment, including all patients carrying the low-expression *CR1-L* allele. CH50 and sC5b-9 assays demonstrate that the persistent low-level hemolysis identified in our treated patients is not a consequence of incomplete C5 blockade, supporting that this hemolysis, as has been suggested previously, results from the extravascular removal of C3 opsonized PNH erythrocytes. We also show that continuous alternative pathway activation in eculizumab-treated individuals carrying the *CR1-L* allele results in abnormally decreased levels of C3 in plasma that could, potentially, increase their susceptibility to bacterial infections. Finally, we encourage a routine evaluation of free eculizumab levels and terminal pathway activity to personalize eculizumab administration.

1. Introduction

Paroxysmal Nocturnal Hemoglobinuria (PNH) is a disorder caused by the proliferation of hematopoietic stem cells carrying, in most of the cases, a somatic mutation in the gene *PIG-A*, which is necessary for the biosynthesis of the glycosil phosphatidyl-inositol (GPI) anchor. As a consequence, PNH erythrocytes (PNH-E) lack, among other proteins, the complement regulators decay accelerating factor (DAF; CD55) and membrane inhibitor of reactive lysis (MIRL; CD59) and become susceptible to complement-mediated intravascular hemolysis (Wilcox et al., 1991; Takeda et al., 1993; Miyata et al., 1993). PNH is effectively treated with eculizumab, a monoclonal antibody that blocks C5 cleavage, impeding activation of the lytic pathway and membrane attack complex (MAC) formation (Thomas et al., 1996; Rother et al., 2007; Hillmen et al., 2004). Eculizumab treatment prevents intravascular hemolysis, which abolishes thrombotic events, the main cause of death in PNH, and results in marked improvement of all clinical parameters and the quality of life of the patients (Hillmen et al., 2006; Brodsky et al., 2008). Despite the unquestionable benefits of eculizumab, the responses to treatment are reported heteroge-

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neous, with most treated patients presenting signs of continuous low-level hemolysis and 25–35% of them still requiring red blood cell (RBC) transfusions (Brodsky et al., 2008; Luzzatto et al., 2011; Kelly et al., 2011). In addition to lytic pathway dysregulation, absence of CD55 and CD59 in PNH-E also impairs regulation of the complement alternative pathway, a situation that persists under eculizumab treatment and that results in intense deposition of activated C3 fragments on the PNH-E surface (Logue et al., 1973). As a consequence of the accumulation of C3 opsonized PNH-E, several eculizumab-treated patients become direct Coombs-test positive (Roth et al., 2010; Hill et al., 2010). C3 opsonized PNH-E are susceptible of extravascular clearance through the reticuloendothelial system in the liver and the spleen (Jaffe et al., 1976; Ross and Lambris, 1982; Lin et al., 2015). This alternative removal mechanism of PNH-E, which is unmasked by the blockade of the lytic pathway-mediated intravascular hemolysis of the PHN-E by eculizumab, is thought to be the cause of the on-going residual hemolysis that present many treated PNH patients (Hill et al., 2010; Risitano et al., 2009). Critically, it has also been found that the intensity of complement deposition in PNH-E inversely correlates with the levels of the complement regulator CR1 (CD35) on erythrocytes, which are determined by genetic variants at the *CR1* locus (de Cordoba and Rubinstein, 1986; Wilson et al., 1986); individuals carrying the low expression allele *CR1-L* being particularly susceptible to experience extravascular hemolysis under eculizumab treatment (Rondelli et al., 2014). In addition to this extravascular removal of heavily opsonized PNH-E, one study has recently indicated that the residual low-level hemolysis could, in some patients, be related to incomplete C5 blockade and recommended close supervision of free eculizumab levels and terminal pathway activity to prevent this possibility (de Latour et al., 2015).

Here, we searched for correlations between hemolysis parameters, complement determinations (including *CR1 H/L* genotypes) and plasma levels of free eculizumab in our cohort of eculizumab-treated patients. We show that most eculizumab-treated patients presented signs of low-level hemolysis. Importantly, this residual hemolysis has no clinical manifestations in our patients. We also show that evaluation of the free eculizumab levels and terminal pathway activity may help to improve rationalization of the eculizumab administration and that eculizumab-treated individuals carrying the *CR1-L* allele may require special attention as they may be predisposed to present more severe disease presentations and susceptibility to infections.

2. Patients, materials and methods

2.1. Subjects

The studies reported here have Institutional Review Board's approval. Informed consent was provided to all individuals participating in the study, according to the Declaration of Helsinki. The study group included 12 patients who were diagnosed of having PNH using flow cytometry techniques, as reported previously (Munoz-Linares et al., 2014). The average age of the patients was 48 years. Eleven were classified as classic-PNH and one as aplasia anemia-PNH, according to the classification of Nakakuma as amended by Parker (Nakakuma et al., 1995; Parker et al., 2005). All patients in our series have received eculizumab because of severe hemolysis, PNH clone granulocytes percentage > 45% and severe symptoms attributable to the disease, causing serious impairments in their quality of life. All patients included in the study have been receiving eculizumab for more than one year (ranging between 1yr and 8yr; mean 4.3 ± 2.1yr). Eculizumab was administrated intravenously following standard guidelines. In all cases, transfusion independence was achieved at the beginning of treatment with disappearance of the symptomatology attributable to the disease. Nine patients have always received 900 mg of the drug every 14d and three patients, due to a breakthrough hemolysis, required a dosing increase that have been maintained since then at 1200 mg every 2wk. All 12 patients have received prophylactic vaccination with meningococcal tetravalent vaccine and are revaccinated every 2yr. Since the beginning of 2013, all patients have received ciprofloxacin 500 mg/d orally (or penicillin oral 400 mg/12 h). In the last months all the patients has been vaccinated with anti-meningococcus B (Bexsero).

We collected samples from all patients treated during 4 weeks (two eculizumab cycles), at three different points during treatment; before and after eculizumab administration and in the intermediate week between two eculizumab cycles. Parameters, such as levels of lactate dehydrogenase (LDH), bilirubin, % of reticulocytes, hemoglobin and Coombs-test data were determined only in the samples collected before the eculizumab administration and in the intermediate week. None of the patients had clinical complications during the length of the study.

2.2. Genotyping

Genomic DNA was prepared from peripheral blood cells of all individuals included in these studies according to standard procedures (Miller et al., 1988). Patients were genotyped for the three polymorphisms in *CR1* gene that allow discrimination of the *CR1-H* and *CR1-L* alleles by automatic DNA sequencing of PCR amplified fragments. To amplify a fragment that includes the *Hind*III RFLP (intron 27) we used the forward primer 5′-GGGTTCTTGCTCTTGACTTC-3′ and the reverse primer 5′- GAATGCTGGACTGTCTTGC-3′. To amplify the region that includes the H1208R (exon22) site we used the forward primer 5′-CCTTGTGCTAGGGAGAATTG-3′ and the reverse primer 5′-CCAGAGGTTAATCTCCCTG-3′ and for the P1827R (exon 33) we used the forward primer 5′-TCCAGGAACACTGTCTTTG-3′ and the reverse primer 5′-TGACAGTTACAGCAAAGCC-3′. Patients were also genotyped for polymorphisms in the C5 exon including the R885H polymorphism associated with poor response to eculizumab treatment (Nishimura et al., 2014). To amplify this region we used the forward primer 5′-GCAGGAGAATTGCTTGAATC-3′ and the reverse primer 5′-GCACGATTTCAGACTTACAGAA-3′. Automatic sequencing was performed in an ABI 3730 sequencer using a dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA.).

2.3. Flow cytometry

Erythrocytes from PNH patients were harvested by centrifugation, washed with PBS several times until the supernatant remained clear and stored for up to a week in ACD-A buffer at 4 °C. C3 deposition on erythrocytes and percentage of PNH-E were determined simultaneously by 2-color flow cytometry. Briefly, a 0.4% suspension of erythrocytes was incubated with a rabbit polyclonal anti human C3 antibody (in house) that recognizes all C3, C3b, iC3b and C3dg at 1 μg/mL in PBS for 30 min at room temperature (RT). Then we incubated the erythrocytes with an anti-rabbit IgG antibody labeled with Alexa 488 (Lifetechnologies) 1 μg/mL and with a mouse monoclonal anti-CD59 labeled with R-Phycoerythrin (Sigma-Aldrich) used in 1:50 dilution for 30 min at RT to identify PNH-E. FLAER detection of the GPI anchor in granulocytes was performed as described previously (Ahluwalia et al., 2014).

2.4. Free eculizumab levels

Levels of free eculizumab circulating in the plasma of PNH patients were determined by ELISA, essentially as described by Peffault de Latour et al. Wells were coated with C5 (10 μl/mL in PBS)