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Polymorphism of the complement receptor 1 gene correlates with hematological response to eculizumab in patients with paroxysmal nocturnal hemoglobinuria

Running title: CR1 polymorphism and response to eculizumab in PNH

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Abstract

Complement blockade by eculizumab is clinically effective in hemolytic Paroxysmal Nocturnal Hemoglobinuria. However, the response is variable and some patients remain red blood cell transfusion-dependent. In 72 patients with hemolytic Paroxysmal Nocturnal Hemoglobinuria on eculizumab we tested the hypothesis that response may depend on genetic polymorphisms of complement-related genes. We found no correlation between the Complement Component C3 genotypes and the need for blood transfusion. On the other hand, we found a significant correlation with the HindIII polymorphism of a complement regulatory gene, the Complement Receptor 1 (CR1) gene. At this locus two co-dominant alleles are known, of which H (common) is associated with high expression, whereas L (rare) is associated with low expression of CR1 on red cells. Patients who still need blood transfusion on eculizumab were 18% among H/H homozygotes, 33% among H/L heterozygotes, and 68% among L/L homozygotes ($P=0.016$). Thus, patients with Paroxysmal Nocturnal Hemoglobinuria who have the L/L genotype are 7 times more likely to be sub-optimal responders to eculizumab. Both in vitro and in vivo we found that the CR1 HindIII genotype correlates with the abundance of paroxysmal nocturnal hemoglobinuria red cells that have bound C3, and with the kinetics of C3 biding. These results are consistent with the notion that by affecting C3 binding the CR1 genotype influences the response to eculizumab treatment, and this emerges as a novel example of pharmacogenetics.
Introduction

Blockade of the distal complement pathway through the use of eculizumab, a monoclonal antibody against Complement Component 5 (C5), has been a major advance in the clinical management of paroxysmal nocturnal hemoglobinuria (PNH).\(^1\), \(^2\) Eculizumab effectively controls in PNH patients intravascular hemolysis and its direct consequences, which results in remarkable clinical improvement.\(^1\), \(^3\) The natural history of the disease, which had already improved before the introduction of eculizumab (compare Hillmen et al., 1995\(^4\) with deLatour et al., 2008\(^5\)), may have improved further with the use of this agent.\(^6\) However, the hematological response to eculizumab is variable. Indeed, in some patients who were not transfusion-dependent the average hemoglobin level increases; the majority of patients who were transfusion-dependent become transfusion-independent, with or without an increase in their average hemoglobin level; but some patients remain transfusion-dependent, even though their transfusion requirement may decrease.\(^7\), \(^8\) In addition, in almost all PNH patients on eculizumab a significant fraction of GPI-negative red blood cells (RBC) are opsonized by Complement Component 3 (C3) (with the previously negative Coombs test becoming positive); and the red cells so opsonized may undergo extravascular hemolysis.\(^7\) This phenomenon, testified also by a persistent reticulocytosis, may not be clinically relevant in most patients; however, in some patients it may limit significantly the hematological benefit from eculizumab. In fact, 25-35% of patients still need RBC transfusions;\(^2\), \(^6\), \(^8\), \(^9\) and the size of the fraction of their own red cells with bound C3 seems to correlate with transfusion requirement.\(^7\), \(^10\)

In view of the above, we have hypothesized that one determinant of the variability of response to eculizumab might be the levels of factors that can regulate complement activation. In order to test this hypothesis we have analyzed genetic polymorphisms of
complement-related genes: specifically, polymorphic alleles of the C3 gene and of the Complement Receptor 1 (CR1) gene.
Methods

Patients. 72 patients with hemolytic PNH who had received eculizumab treatment were analyzed after a median follow-up of 52 months (range: 11-98 months). Patients with evidence of bone marrow failure (reticulocytes ≤60,000/µL, platelets ≤50,000/µL, neutrophils ≤1000/µL) have not been included in this series because this condition may affect the clinical response to eculizumab regardless of how well hemolysis is controlled. Peripheral blood samples were collected for clinical testing, flow cytometry (GPI-molecules and C3-binding) and genotyping after the patients signed an informed consent approved by the respective Institutional Review Board. As a criterion of response to eculizumab we used the one that is most stringent and objective: namely RBC transfusion at any time after the first 6 months on eculizumab. Of the 72 patients 60 were transfusion-dependent before eculizumab: of these 60, those who on eculizumab received no further transfusion during follow-up have been classified as good responders; those who on eculizumab received one or more RBC transfusion at any time during follow-up have been classified as suboptimal responders. Twelve patients started eculizumab before having received any RBC transfusions. Of these, the 10 patients who on eculizumab remained transfusion-independent have been classified as good responders because their hemoglobin level increased, on average, by 2.2 G/dL; the remaining 2 patients required RBC transfusions on eculizumab and have been classified as sub-optimal responders. Only one patient, who had hemolytic PNH at the time of starting eculizumab and who had been already classified as a sub-optimal responder became aplastic during follow-up.
Genotyping. The polymorphism rs2230199 C>G of the C3 gene was investigated by a newly designed tetra-primer amplification refractory mutation system-polymerase chain reaction method. Three polymorphisms of the CR1 gene were genotyped by restriction fragment length (RFLP) analysis: HindIII RFLP (intron 27); His1208Arg (exon 22); Pro1827Arg (exon 33).

Kinetics of C3 binding in vitro. RBC and sera were promptly separated from freshly collected peripheral blood from PNH patients. RBC were then incubated with sera containing eculizumab as previously described. Briefly, a 2% suspension of RBC from PNH patients was incubated at 37°C with a pool of AB0-compatible sera from patients on eculizumab, and complement alternative pathway was activated by mild acidification (HCl 0.016 M). At serial time points (15’, 30’, 60’, 120’) after complement activation the fraction of GPI-negative RBC with newly bound C3 fragments was measured by flow cytometry (AccuriC6, Becton Dickinson, NJ, USA) after staining with anti-CD59 Alexa647 (Mem43, Serotec, UK) and anti-C3d-neoantigen (A250, Quidel, CA, USA); secondary staining with PE polyclonal rabbit-anti-mouse antibodies (Dako Cytomation, Denmark).

Statistical analysis. Hardy-Weinberg equilibrium was tested with Pearson’s χ² test by the Finetti program (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl). The association between genotypes and response was tested by the χ² test for trend, because these polymorphisms affect gene expression (or gene product activity), and for each one of them the level of expression in heterozygotes is intermediate with respect to both homozygotes. Mann-Whitney and Kruskal-Wallis tests were used for continuous variable as appropriate. All statistical tests were two-sided; statistical significance was accepted for P<0.05.
Results and Discussion

C3 is central in the complement system. The single nucleotide polymorphism rs2230199 C>G of the C3 gene is responsible for the allelic electrophoretic variants slow (common allele) and fast (rare allele). This polymorphism of C3 influences the complement alternative pathway activity and it is known to be associated with certain complement-mediated disorders. In 72 hemolytic PNH patients on eculizumab the frequencies of the slow and fast alleles of the C3 polymorphism rs2230199 were in Hardy-Weinberg equilibrium. We found no correlation between response to eculizumab and C3 genotype (Table 1. P=0.939).

The distribution of the levels on red cells of CR1, an important cell surface complement regulatory protein, is trimodal in the general population. This variability of expression is determined by two co-dominant alleles: H (high expression) and L (low expression). In Europe and in Asia, but not in Africa, these two alleles are associated with a HindIII RFLP, which is in strong linkage disequilibrium with a number of other polymorphisms in the CR1 coding sequence, including His1208Arg and Pro1827Arg. Although CR1 is not a GPI-linked molecule, it has been suggested that it may play a role in PNH.

In our series of 72 PNH patients the genotype frequencies of these CR1 polymorphisms are in Hardy-Weinberg equilibrium. Since only 5 of our patients are of African descent, a high level of linkage disequilibrium among the 3 polymorphisms was expected: in fact, only in 3 patients the common CR1 HindIII RFLP allele was not associated with the common CR1 Pro1827Arg allele, and in only 1 of these it was not associated with the common CR1 His1208Arg allele. Thus, the frequency of the rare alleles were almost identical: 0.31 for the HindIII RFLP and for His1208Arg; 0.29 for Pro1827Arg. When we considered the response to eculizumab of individual patients, we found that the proportion of sub-optimal
responders was significantly higher in patients who were heterozygotes for the CR1 HindIII polymorphism (genotype H/L); and even higher in those who were homozygous for the rare allele (genotype L/L) (Table 1. \( P=0.016 \)). This correlation, as expected, is also significant for the two closely linked polymorphisms His1208Arg and Pro1827Arg (Table 1). It is important to note that the assessment of response to eculizumab, on which this result is based, was substantiated over a follow-up period with a median of over 4 years (minimum 1 year).

In principle the CR1 HindIII polymorphism might influence blood transfusion requirement in PNH regardless of eculizumab therapy. However, this is not the case: in patients with CR1 HindIII genotypes H/H, H/L and L/L the mean pre-eculizumab hemoglobin levels (G/dL) were 8.3, 8.2 and 8.0 respectively (\( P=0.76 \)); and packed RBC units/month were 0.6, 0.8 and 0.8 respectively (\( P=0.38 \)). Interestingly, the percentage of patients not requiring RBC transfusions before eculizumab was higher (33\%) among L/L genotype patients than among H/H and H/L genotype patients (15\%). The different response of patients with the three genotypes to eculizumab was not related to a difference in the control of intravascular hemolysis: in fact, the levels of LDH were similar in good responders and in sub-optimal responders: 1.05 and 1.18 times the normal value respectively (\( P = 0.11 \)). In addition, patients on eculizumab with CR1 HindIII genotypes H/H, H/L and L/L have similar levels of LDH: 1.05, 1.13 and 1.10 times the normal value respectively (\( P = 0.80 \)). Thus, in this series the CR1 HindIII genotype is not associated with significantly different clinical features, but rather with the way patients respond to eculizumab.

Our findings might be explained by considering the multiple functions of CR1 within the complement system. Indeed, by binding C3b and C4b CR1 enhances the decay of the C3 and C5 convertases; and since CR1 is a cofactor of Factor I it can also help inactivating C3b and C4b, thus tuning both the alternative and the classical complement
activation pathways.\textsuperscript{29, 30} In addition, CR1 plays a role in the clearance of immune complexes and in phagocytosis.\textsuperscript{31-33} Thus, the lower levels of CR1 expression on the red cell surface associated with the H/L and L/L genotypes\textsuperscript{22} are expected to result in increased complement activation and C3 binding on PNH red cells.

In order to test this hypothesis we have investigated \textit{in vitro} the kinetics of C3 binding to GPI-negative red cells\textsuperscript{13, 14} from patients with the three CR1 HindIII genotypes. When GPI-negative red cells were exposed to activated complement in the presence of eculizumab, we detected promptly C3+ PNH red cells, the percentage of which increased with time.\textsuperscript{14} The rate of this increase was highest with red cells with the L/L genotype, lowest with red cells with the H/H genotype, and intermediate with red cells with the heterozygous genotype (Figure 1). These \textit{in vitro} findings suggest that the density of CR1 on the surface of red cells modulates the binding of C3 fragments to the GPI-negative red cells when C5 is blocked by eculizumab. These data are also in good agreement with the observation that in our patients on eculizumab the CR1 HindIII genotypes correlate with the fraction of GPI-negative red cells with bound C3 \textit{in vivo}: H/H ($n=20$) 21.4 ± 19.9\%, H/L ($n=16$) 29.1 ± 16.2\% and L/L ($n=4$) 46.3± 19.5\% ($P=0.032$).

During eculizumab treatment it may occur that abrupt complement activation by intercurrent infection or inflammation sporadically overcomes C5 blockade; resulting, especially in patients with low levels of CR1, in occasional episodes of intravascular hemolysis, irrespective of the plasma level of eculizumab.\textsuperscript{9} However, more commonly, when C5 is effectively blocked, low CR1 levels on red cells would continuously facilitate and increase C3 binding on red cell surface because the decay of C3 convertase is slower. Thus, there will be an increased proportion of C3-opsonized red cell that can be removed by phagocytosis, resulting in PNH patients with these CR1 genotypes, in a higher rate of chronic extravascular hemolysis.\textsuperscript{9} This relationship between the response to
eculizumab and the CR1 genotype is in keeping with our current understanding of the pathophysiology of PNH on eculizumab treatment.\textsuperscript{7,9}

Given the small number of L/L homozygotes in our series we must be guarded in drawing definitive conclusions: larger prospective studies will be needed. Nevertheless, the concordance between clinical, biological and \textit{in vitro} data indicates that the CR1 polymorphism may predict the hematological response to eculizumab. Patients on eculizumab, even when they remain transfusion-dependent, benefit considerably from this treatment in terms of reduced risk of thrombosis and of subjective symptoms, and thus in their quality of life. Therefore, although our data indicate that PNH patients with the CR1 HindIII RFLP L/L genotype are 6.7 times more likely to be sub-optimal responders to eculizumab than patients with the H/H genotype, this does certainly not disqualify them from eculizumab treatment. Rather, the interaction between the CR1 polymorphism and eculizumab, a novel example of pharmacogenetics may help to explain the pattern of response to eculizumab. This is important, particularly in view of the development of new complement-targeting agents for the management of PNH.\textsuperscript{13}
Authorship and Disclosure

L.L. and R.N conceived the study. T.R. and B.P. performed the genotyping under the supervision of M.D. M.S. helped in genotyping and performed the kinetics studies. P.R. helped in the experimental work. All the authors, with the exception of T.R., B.P., P.R., V.F. and M.D., were in charge of the clinical management of the patients. T.R., A.M.R, M.D., R.P., M.S., V.F., G.S., L.L. and R.N. discussed and interpreted the data. The paper was written by R.N., A.M.R., R.P., G.S. and L.L. and all the authors critically revised the paper and contributed to the preparation of the final version.

R.N. and A.M.R. have served as consultants and have received research grant from Alexion Pharmaceuticals. L.L., G.S., R.P. have served as advisors to Alexion Pharmaceuticals. A.P.I. has received lecture fees from Alexion Pharmaceuticals. The remaining authors declare no competing financial interests.
References


Table 1. Association of C3 gene and of CR1 gene polymorphisms with hematological response to eculizumab.

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Patients, n</th>
<th>Good Responders n (%)</th>
<th>Suboptimal Responders n (%)</th>
<th>OR (CI)</th>
<th>P *</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C3 Slow/Fast</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slow/Slow</td>
<td>47</td>
<td>34 (72.3)</td>
<td>13 (27.7)</td>
<td>1.0</td>
<td>0.934</td>
</tr>
<tr>
<td>Slow/Fast</td>
<td>22</td>
<td>14 (63.6)</td>
<td>8 (35.4)</td>
<td>1.0</td>
<td>(0.4- 2.4)</td>
</tr>
<tr>
<td>Fast/Fast</td>
<td>3</td>
<td>3 (100.0)</td>
<td>0 (0.0)</td>
<td>1.0</td>
<td>(0.2- 5.9)</td>
</tr>
<tr>
<td><strong>CR1 HindIII RFLP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High/High</td>
<td>33</td>
<td>27 (81.8)</td>
<td>6 (18.2)</td>
<td>1.0</td>
<td>0.016</td>
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<tr>
<td>High/Low</td>
<td>33</td>
<td>22 (67.7)</td>
<td>11 (33.3)</td>
<td>2.6 (1.1- 6.0)</td>
<td></td>
</tr>
<tr>
<td>Low/Low</td>
<td>6</td>
<td>2 (33.3)</td>
<td>4 (67.7)</td>
<td>6.7 (1.3- 36.0)</td>
<td></td>
</tr>
<tr>
<td><strong>CR1 His1208Arg</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>His/His</td>
<td>34</td>
<td>28 (82.4)</td>
<td>6 (17.6)</td>
<td>1.0</td>
<td>0.012</td>
</tr>
<tr>
<td>His/Arg</td>
<td>32</td>
<td>21 (34.4)</td>
<td>11 (34.4)</td>
<td>2.7 (1.2- 6.2)</td>
<td></td>
</tr>
<tr>
<td>Arg/Arg</td>
<td>6</td>
<td>2 (33.3)</td>
<td>4 (67.7)</td>
<td><strong>7.26</strong> (1.4-38.8)</td>
<td></td>
</tr>
<tr>
<td><strong>CR1 Pro1827Arg</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro/Pro</td>
<td>36</td>
<td>28 (77.8)</td>
<td>8 (22.2)</td>
<td>1.0</td>
<td>0.054</td>
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<tr>
<td>Pro/Arg</td>
<td>30</td>
<td>21 (70.0)</td>
<td>9 (30.0)</td>
<td>2.1 (0.9- 4.6)</td>
<td></td>
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<tr>
<td>Arg/Arg</td>
<td>6</td>
<td>2 (33.3)</td>
<td>4 (66.7)</td>
<td><strong>4.4</strong> (0.9-21.6)</td>
<td></td>
</tr>
</tbody>
</table>

OR: Odd ratio; CI: 95% confidence limits.
* Mantel-Haenszel $\chi^2$ test for trend
Figure 1. The CR1 genotype is a major determinant of the rate of in vitro C3 binding to GPI-negative red cells. In each patient this time course experiment was carried out twice. H/H: homozygotes for the high expression allele of CR1 HindIII RFLP polymorphism. L/L: homozygotes for the low expression allele of CR1 HindIII RFLP polymorphism. H/L= heterozygotes for CR1 HindIII RFLP polymorphism. Standard deviation is reported for each experimental point. When eculizumab-containing sera are added to red cells, the slope of novel C3 binding to GPI-negative red cells is significantly different depending on the CR1 genotype. For the comparison of H/H (n = 6) vs. H/L (n = 3), P = 0.0425; for the comparison H/L (n = 3) vs. L/L (n = 2), P = 0.0173; for the comparison H/H (n = 6) vs. L/L (n = 2), P < 0.0001. Given a certain CR1 genotype these differences were not the result of eculizumab treatment: indeed, in 3 patients with the H/H genotype who were on eculizumab the rate of C3 binding to GPI-negative red cells was very similar to that seen in 3 patients with the H/H genotype who were not on eculizumab (P = 0.87).