Paroxysmal nocturnal hemoglobinuria and eculizumab*

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doi:10.3324/haematol.2009.017848

**This paper is dedicated to the dearest memory of Bruno Rotoli: for us and for many others a trusted colleague, inspiring mentor, and long-time friend.*

(Related Original Articles on pages 567 and 574)

Paroxysmal nocturnal hemoglobinuria (PNH) is enough of a tongue-twister for patients and doctors alike and we all teach the students that PNH is a disorder characterized by the triad of intravascular hemolysis, venous thrombosis and cytopenias.[1-3] But in this journal, founded by Adolfo Ferrata in the twenties, it seems appropriate to remember that, in the Italian literature, the full name coined for PNH in 1928 by Ettore Marchiafava was even more articulate: *paroxysmal nocturnal haemoglobinuria with perpetual haemosiderinuria*.[4] The last qualification is not trivial, because even today the accesses of hemoglobinuria, most distressing for the patient, may tend to obscure the fact that in PNH hemolysis goes on all the time: macroscopic hemoglobinuria merely reflects its exacerbations.[5]

That in PNH hemolysis was complement-dependent has also been known for a long time (Figure 1): indeed, the acidified serum (or Ham-Dacie) test, based on complement-mediated lysis of PNH cells *in vitro*, has for half a century been the gold standard for the diagnosis of PNH,[7, 8] until flow cytometry came into its own.[9, 10] Therefore stopping hemolysis by blocking complement was a logical idea: it did not seem easy to achieve this safely *in vivo*. But the idea has become a reality through the advances of biotechnology, when Alexion developed a humanized anti-C5 monoclonal antibody, eculizumab,[11] which has proved highly effective in the control of intravascular hemolysis in patients with PNH:[12-14] a huge step forward in the treatment of a disorder that until now could be cured only by hematopoietic stem cell transplantation.[15-17]

PNH is associated with the most vicious acquired thrombophilic state known to medicine, with nearly one-half of untreated patients sooner or later affected: the consequences are potentially devastating,[5, 18-20] and it is humbling to admit that we still don't know the mechanism.[21] There are two main questions: (a) what triggers a major thrombotic event in an individual patient, sometimes years into the course of the disease; (b) why are about one-half of the patients spared this complication. Until we know the answers to these questions, they pose a difficult management problem. If a patient with PNH is not given anti-coagulant prophylaxis, there is a significant risk that sooner or later that patient may develop a serious thrombotic complication.[5, 22-24] On the other hand, if all patients with PNH are given anticoagulant prophylaxis the added burden is significant: in addition, it is unnecessary for one-half of them, it does not completely prevent thrombosis, and it entails the risk of serious hemorrhage.[25] In view of this, it has come as an added bonus that patients on eculizumab therapy have a substantially lower risk of thrombosis.[26]

In this issue of Haematologica, a paper by Gerard Socié's group[27] deals with how eculizumab may influence venous thrombosis in PNH, and Peter Hillmen's group provide data[28] on the recently developed notion[29] that PNH patients on eculizumab show evidence of extravascular hemolysis, which may be an important determinant of the clinical response to this new therapy.

Socié *et al.* have systematically investigated patients on eculizumab and they have found that, compared to pre-treatment values, they tend to have lower levels of coagulation factors such as von Willebrandt factor, of by-products of coagulation such as D-dimers, and also of endothelial cell molecules such as VCAM. These findings do not resolve the issue as to whether eculizumab prevents thrombosis indirectly by curbing intravascular hemolysis (which perhaps triggers thrombosis through the release of thromboplastin-like substances), or whether it acts directly by blocking C5 (which in turn might activate platelets and/or the coagulation pathway)[30]. However, the data provide evidence that in patients on eculizumab there are changes in objective parameters that may correlate with the risk of thrombosis, and this may also help to unravel the puzzle of thrombophilia in PNH.

The paper by Hillmen *et al.*[28] reports that in 21 out of 31 PNH patients on eculizumab the direct antiglobulin test (DAT) was positive for C3 on red cells (one was also weakly positive for IgG), whereas only one had been DAT-positive before treatment; in contrast, out of 39 PNH patients not on eculizumab only 2 were DAT-positive for C3. These data are a clear confirmation of published work on a separate series of PNH patients[29] which had shown that: (i) the C3-positive red cells belong entirely to the PNH red cell population; (b) the proportion of C3-positive red cells correlated with the reticulocyte count; (iii) some patients with a high proportion of C3-positive red cells had decreased *in vivo* survival of ^{51}Cr-labeled red cells with excess counts on spleen and liver, proving formally that they have extravascular hemolysis. Hillmen *et al.* provide further confirmation that these findings have important clinical implications: indeed, the mean hemoglobin level was significantly higher in patients who, on eculizumab, remained DAT-negative. Moreover, of the patients who remained DAT-negative only one (10%) has needed blood transfusion, whereas of those who developed a positive C3 DAT on eculizumab, 76% have needed at least one blood transfusion.

These data illustrate a *leitmotiv* in the history of medicine: any new therapy, even when it proves remarkably successful, as is the case for eculizumab, may have limitations; at the same time, a new therapy can give us new insights into the pathophysiology of the disease it treats. With respect to the risk of thrombosis, it is unlikely that a prospective clinical trial

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may be conducted. Therefore, it will be important to confirm the current notion that eculizumab is protective,[26, 31] whatever the mechanism, by independent datasets with sufficient longitudinal follow-up. In addition, in the case of a patient on eculizumab who has had a previous thrombotic event, a tricky problem is whether prophylactic anticoagulation[32] can or ought to be discontinued. Again, it is unlikely that this issue will be addressed by an *ad hoc* clinical trial; however, observation of patients in whom anticoagulation has been withdrawn because of hemorrhagic complications may provide hints for an educated case by case clinical decision.

As for opsonization of GPI(-) red cells by C3, this is probably favored by CD55 deficiency. In untreated patients with PNH, the red cells that have bound C3 will selectively activate C5 and will hence succumb to the membrane attack complex (MAC). For this reason we practically never see a positive DAT in a newly diagnosed PNH patient (Figure 1B). In contrast, once C5 is blocked by eculizumab, GPI(-) C3-coated red cells have a chance to accumulate, and they will become prey to phagocytosis by macrophages (Figure 1C). Thus, it is likely that the phenomenon of C3 binding is simply being unmasked by eculizumab; but the consequent extravascular hemolysis is

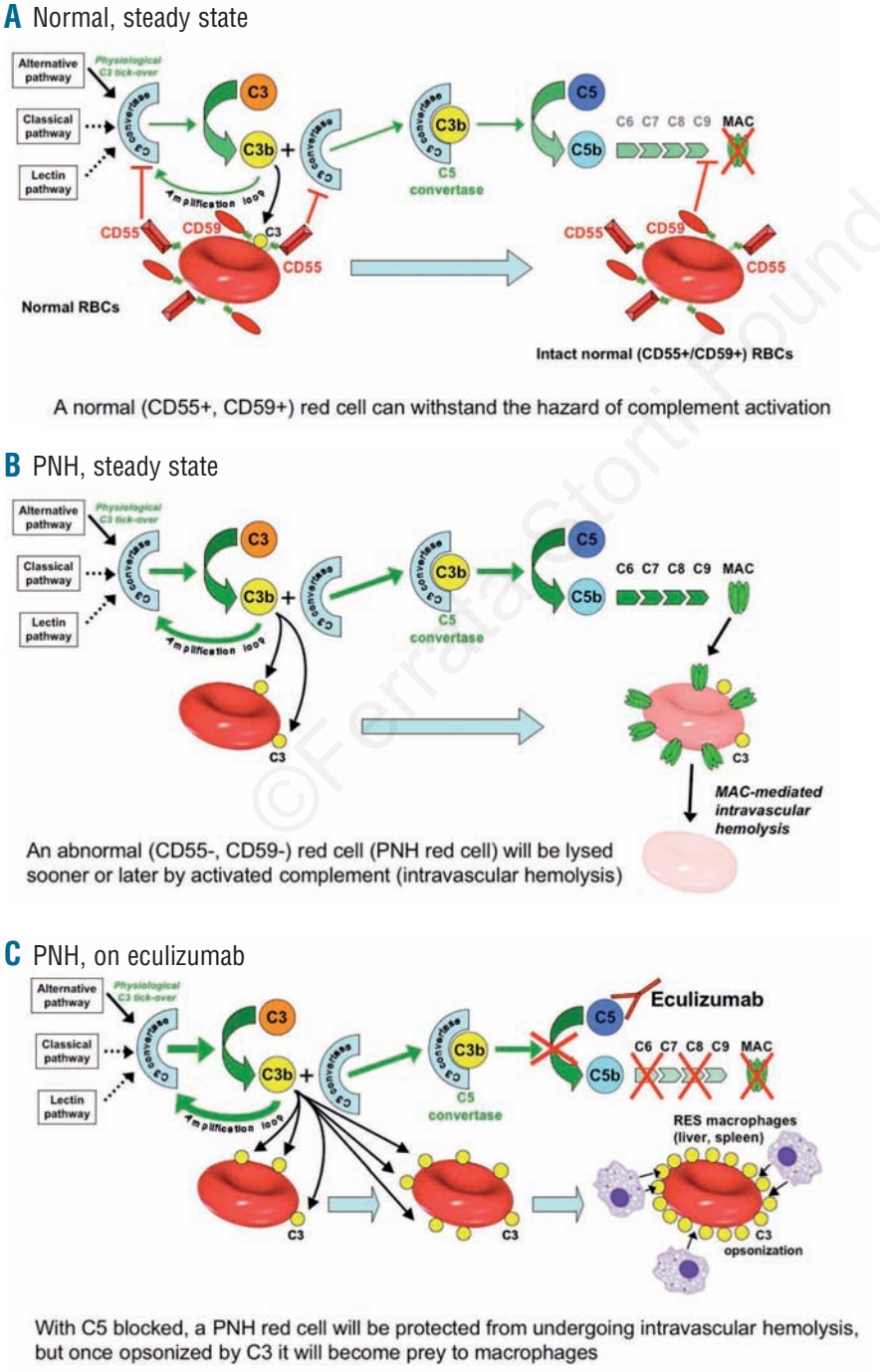

Figure 1. The complement cascade regulation and erythrocytes. (A) Normal erythrocytes are protected from complement activation and subsequent hemolysis by CD55 and CD59. These two proteins, being GPI-linked, are missing from the surface of PNH red cells as a result of a somatic mutation of the X-linked *PIG-A* gene, that encodes a protein required for an early step of the GPI molecule biosynthesis.[6] (B) In the steady state, PNH erythrocytes suffer from spontaneous (tick-over) complement activation, with consequent intravascular hemolysis through formation of the MAC; exacerbated hemolysis will result from activation of extra complement through the classical pathway. (C) On eculizumab, PNH erythrocytes are protected from hemolysis from the inhibition of C5 cleavage; however, upstream complement activation may lead to C3 opsonization and possible extravascular hemolysis. Here "C3 convertase" represents either the C3bBb complex (alternative pathway) or the C4b2a complex (classical and lectin pathways).

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really a new phenomenon. Since the majority of patients on eculizumab have a persistent (though usually mild) reticulocytosis, this phenomenon is probably the rule rather than the exception: however, fortunately in many cases it seems to have little or no clinical relevance.

The very trial that proved the success of eculizumab in PNH patients with severe hemolysis produced a dichotomy between those who became transfusion-independent and those who did not.[13, 14] It seems likely that this dichotomy results at least to a large extent from how many of their PNH red cells bind C3 and we must find out what factors influence the quantity and/or quality of this binding. In this respect, a major question is how to manage C3-mediated extravascular hemolysis in those patients in whom this creates a clinical problem, especially in view of the attendant risk of iron overload, a complication previously unknown in PNH. It is questionable whether corticosteroids might be effective but many patients with PNH have already suffered from the side effects of chronic administration of these agents, and it would not be desirable to subject them to these once again. Other forms of immunosuppression and intravenous Ig may or may not work but they would certainly be an added burden. Splenectomy has been reported effective in one patient[33] but since it increases the risk of infection and thrombosis one would never recommend it as a standard measure. Speculative approaches for the future might include: (a) targeting the complement cascade upstream of C5; (b) interfering with the interactions between C3-opsonized red cells and macrophages; (c) targeting the interaction between C3 and the red cells themselves. In this respect, one of us has reported recently[34] that TT30, a recombinant human protein consisting of the fusion of the iC3b/C3d-binding region of complement receptor 2 with the functional domains of the complement regulator factor H, is able to prevent hemolysis of PNH red cells *in vitro*. In essence, TT30 seems to surrogate the function of CD55, lacking on PNH red cells, by inhibiting C3 activation and deposition on red cells, thus blocking the formation and activity on their surface of the C3 convertase. Of course, it is unlikely that we can safely get rid of complement altogether, and it remains to be seen whether such modes of complement blockade will be as safe as blocking just C5.

In conclusion, with the introduction of eculizumab in the first decade of this century we have for the first time a specific medicine for PNH. For many patients this has meant having PNH without hemoglobinuria, without numerous unpleasant symptoms, without depending on blood transfusion, and with a much reduced risk of venous thrombosis. For the rest of the patients, in the next decade we have to find out how best to control extravascular hemolysis. For all patients, we are still left with the challenge of developing a form of medical treatment that will not only control but actually eradicate PNH.

Lucio Luzzatto is a senior hematologist whose first paper on PNH was published exactly 40 years ago [Oni SB, Osunkoya BO, Luzzatto L. Paroxysmal nocturnal hemoglobinuria: evidence for monoclonal origin of abnormal red cells. Blood. 1970 Aug;36(2):145-52]; he has personally seen PNH patients in all 5 continents. Currently he is Scientific Director of the Istituto Toscano Tumori. Antonio M. Risitano, MD, PhD, is a hematologist in the Hematology Division of the Federico II University of Naples. His work is focused on clinical aspects and pathophysiology of bone marrow failure syndromes, including aplastic anemia and paroxysmal nocturnal hemoglobinuria. Rosario Notaro is a hematologist in charge of one of the Research Units of the Core Research Laboratory of the Istituto Toscano Tumori. Professor Luzzatto and Dr. Notaro are very grateful to Professor Alberto Bosi for having hosted PNH clinical trials and current PNH clinical activities.

Dr. Risitano reports receving lecture fees and grant support from Alexion Pharmaceuticals. Dr. Notaro reports receiving lecture and consulting fees and grant support from Alexion Pharmaceuticals and Taligen Therapeutics.

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Molecular basis and clonal evolution of myeloproliferative neoplasms

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doi:10.3324/haematol.2009.019570

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Clonal hematopoiesis is a key feature of myeloproliferative neoplasms (MPN), a hematopoietic stem cell disorder comprising three disease entities: polycythemia vera (PV), essential thrombocytosis (ET) and primary myelofibrosis (PMF). Although the clinical characteristics of these three diseases are quite distinct, they do share genetic mutations that drive clonal myeloproliferation, which is their common defining feature. Continual technological advances have led to the identification of increasing numbers of genetic defects in MPN patients. In this issue of Haematologica, Stegelmann *et al.*[1] present their results from a genome-wide single-nucleotide polymorphism (SNP) profiling study in a large cohort of MPN patients that significantly furthers our understanding of the molecular causes and diversity of MPN.

Genetic defects driving clonality in myeloproliferative neoplasms

The identification of activating mutations in the gene coding for the tyrosine kinase JAK2 (JAK2-V617F and JAK2 exon 12 mutations) in almost all PV patients and in a large percentage of ET and PMF patients,[2-7] led to a series of follow-up studies to investigate their functional consequences.[8] Nevertheless, how a single mutation can contribute to the pathogenesis of three phenotypically distinct disease entities is still largely unclear. One study, using a JAK2-V617F transgenic mouse model, suggested that the disease phenotype could be determined by the ratio of mutant JAK2 to wild-type protein.[9] However, there is growing evidence for a more complex mechanism of MPN phenotype initiation. Observations of strain-specific differences in JAK2-V617F-associated disease phenotypes between Balb/c and C57Bl/6 mice suggest an involvement of germ line features in the resultant phenotype.[8] However, besides JAK2 mutations and other oncogenic events targeting the JAK-STAT pathway (such as mutations in the *MPL* gene, coding for the thrombopoietin receptor and truncations of the erythropoietin receptor),[8] there are still many uncharacterized somatic cytogenetic lesions present within the MPN clone, harboring a series of candidates for both the induction of clonal expansion and the MPN phenotype decision.

TET2 and CBL mutations in myeloproliferative neoplasms

Classical cytogenetic methods made it possible to detect large genetic aberrations in patients with specific diseases which revealed cytogenetic lesions usually containing a large number of genes. Recurrent defects found in MPN, such as the deletions on chromosome 20q (del20q) and 13q (del13q), are thought to harbor important tumor sup-

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