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In this Issue...*Paroxysmal Nocturnal Hemoglobinuria*

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare clonal hematopoietic stem cell disorder.¹ The disease has an incidence of roughly 2-5 per million in the United States and is caused by a somatic mutation of a gene termed PIG-A. The PIG-A gene product is responsible for the first step in the biosynthesis of glycosylphosphatidylinositol (GPI) anchored proteins; hence, the PNH stem cell and all of its progeny have a marked deficiency or absence of all GPI-anchored proteins.² Two GPI-anchored proteins, CD55 and CD59, are complement regulatory proteins and their absence explains the complement mediated intravascular hemolysis and possibly the propensity for thrombosis that characterizes PNH.

In this issue we review current literature that describes new assays to diagnose PNH and how the size of the PNH clone correlates with risk for thrombosis; report on a novel humanized monoclonal antibody (eculizumab) that mitigates hemolysis in PNH by blocking the terminal portion of the complement cascade; and discuss recent investigations into the mechanism of clonal dominance in PNH.

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+ **Commentary and Reviews by:**
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Commentary

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PNH is a clonal hematopoietic stem cell disorder that may occur *de novo* or evolve from aplastic anemia. Somatic mutations of the X-linked gene PIG-A have been found in all PNH patients examined to date.^{3;4} The classical form of PNH manifests with intravascular hemolysis and hemoglobinuria, and may be accompanied by venous thrombosis, esophageal spasm, and erectile dysfunction. While hemoglobinuria is often the most conspicuous manifestation of the disease, venous thrombosis is the leading cause of death from PNH, with abdominal, cerebral, and dermal veins the most common sites of thrombosis. The intravascular hemolysis that occurs in PNH leads to high levels of free hemoglobin in the serum, which may explain both the transient erectile dysfunction in male PNH patients and the esophageal spasm, since free hemoglobin binds with high affinity to nitric oxide (resulting in a relative nitric oxide deficiency at the tissue level).⁵

The diagnosis of PNH has evolved over the last few decades. Before the 1990s, complement-based assays such as the sucrose hemolysis test and the Ham test were used to diagnose PNH. These assays were relatively insensitive and non-specific, especially in patients requiring red cell transfusions, since they were performed on erythrocytes only. Furthermore, these assays were not quantitative. In the early 1990s monoclonal antibodies to GPI-anchored proteins (e.g. anti-CD59 and anti-CD55) began to replace the sucrose hemolysis and Ham test to diagnose PNH.⁶ In the late 1990s GPI anchored proteins were shown to be the receptor for proaerolysin, a bacterial channel forming toxin derived from *aeromonas hydrophila*. Since PNH cells are deficient in GPI-anchored proteins they are resistant to the toxin.⁷ Proaerolysin can be used to select for small populations of PNH cells. In addition, a fluoresceinated proaerolysin variant (FLAER) that binds to the GPI anchor without forming channels can be used in conjunction with flow cytometry to diagnose PNH.⁸ When compared directly, FLAER was shown to be more sensitive and specific for the diagnosis of PNH and appears to give a better estimate of the size of the PNH clone. Studies examining the natural history of PNH are largely based on the complement-based assays (sucrose hemolysis and Ham test);^{9;10} thus, these studies were likely to have excluded patients with relatively small PNH clones and were unable to correlate symptoms and natural history with the size of the PNH clone.

Interestingly, small PNH populations (1 per 50,000 granulocytes) have also been detected in the blood of healthy controls, suggesting that PIG-A mutations are necessary but insufficient to cause disease. More recent data suggest that these PIG-A mutations are polyclonal and do not occur at the hematopoietic stem cell level; hence, they may have no relevance to the pathophysiology of PNH.¹¹ In PNH, PIG-A mutations occur at the level of a hematopoietic stem cell and are usually monoclonal. The mechanism whereby this clone achieves dominance and expands to produce clinical manifestations of PNH is unclear; however, there is now a promising new PNH treatment. Eculizumab is a monoclonal antibody that binds to C'5 and prevents cleavage into C'5a and C'5b, thereby inhibiting the formation of the membrane attack complex.¹² Eculizumab appears to be most effective in patients with classical PNH but not those with overlap aplastic anemia/PNH. It is important to recognize that eculizumab will not cure PNH; by inhibiting the terminal stage of the complement cascade, the drug protects PNH erythrocytes from complement-mediated destruction. The drug appears to decrease the rate of paroxysms and the need for transfusions, but more patients and follow-up will be necessary to determine whether it indeed decreases the risk for thrombosis.

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INVESTIGATING THE NATURAL HISTORY OF PNH

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In order to investigate the natural history of PNH, Moyo et al, performed a retrospective analysis of forty-nine consecutive PNH patients evaluated at Johns Hopkins who were diagnosed using modern diagnostic assays. Of these patients, 44 had their diagnosis confirmed using FLAER. The median age of diagnosis was 34 (range: 6.3 to 80.7) years. Twenty patients presented as classical PNH and twenty-five patients presented as aplastic anemia with small to moderate sized PNH clones; four patients originally diagnosed as aplastic anemia evolved to classical PNH.

The authors found a strong association between the size of the PNH clone (measured as FLAER negative granulocytes) and thrombosis. No patient with less than 61% FLAER negative granulocytes developed a venous thrombosis, whereas 12 of 22 patients (55%) with greater than 61% FLAER negative granulocytes developed venous thrombosis. Using logistic regression the authors found that the odds ratio for thrombosis was 1.64 for a 10% change in the size of the PNH granulocytes clone. Other PNH manifestations that strongly correlated with the size of the PNH clone included abdominal pain, hemoglobinuria, esophageal spasm, and male impotence.

These data, complementing the work of other investigators (notably Hall et al – see [Sources for Additional Information 1](#) at the end of this program) , confirm that the risk for thrombosis and other PNH symptoms related to the nitric oxide “deficiency” in PNH are strongly associated with the size of the PNH clone. In spite of the high risk for thrombosis in PNH patients with large clones, it remains controversial as to whether these patients should receive prophylactic anticoagulation. Since many of these patients have frequent paroxysms of nausea, vomiting, and anorexia, maintaining a therapeutic INR on warfarin is often challenging; furthermore, the platelet count in many PNH patients is moderately to severely depressed, which further increases the risk of anticoagulation. PNH patients with large clones and classical symptoms are also the most likely to benefit from therapeutic strategies, such as eculizumab, that protect cells from complement-mediated lysis. Nevertheless, it remains uncertain whether drugs like eculizumab will decrease the risk for thrombosis.

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SUSTAINED RESPONSE AND LONG-TERM SAFETY OF ECULIZUMAB IN PNH

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Eculizumab is humanized monoclonal antibody that specifically targets and prevents cleavage of the complement protein C5. The drug prevents the generation of C5a and the formation of C5b-9, thereby inhibiting the membrane attack complex caused by the complement cascade; however, early components of the complement cascade, essential for clearance of microorganism and immune complexes, are preserved. In 2004, Hillman et al¹² reported on a 12 week open-labeled study with eculizumab in eleven patients that showed a dramatic reduction in hemolysis, a decrease in the rates of paroxysms, and a reduced need for blood transfusions.

The more recent report by Hill and colleagues examines the results of a 1 year extension study of these original 11 patients. Eculizumab was given at a dose of 900 mg intravenously every 12 to 14 days. A dramatic decrease in hemolysis, paroxysms, LDH, and need for red cell transfusions was maintained throughout the study with no significant adverse events. The paroxysm rate (days with gross evidence of hemoglobinuria per patient each month) decreased from a pre-treatment rate of 3.0 to 0.2 during treatment, and the median transfusion rate decreased from 1.8 U per patient each month before eculizumab to 0.3 U per patient each month (P = 0.001) during treatment.

Eculizumab appears to be highly effective for controlling paroxysms in PNH and preventing the need for frequent blood transfusion in patients with the classical form of the disease (large PNH clone, moderate to severe hemolysis, elevated LDH, elevated reticulocyte count and cellular bone marrow). In patients with significant underlying bone marrow failure (aplastic anemia/PNH) inhibition of complement is less likely to produce a clinical benefit. While eculizumab should prove to be a major advance in the treatment of hemolysis in PNH, it is not a cure; the percentage of PNH red cells actually increases on eculizumab. It is unlikely that eculizumab will prevent the 5% to 10% risk of transformation of PNH into MDS/leukemia, and larger studies with longer follow-up will be required to determine whether eculizumab decreases the risk for venous thrombosis, the leading cause of death from PNH. Results of a double-blinded placebo controlled trial in PNH patients should become available later this year.

1. Hill A, Hillman P, Richards SJ et al. Sustained response and long-term safety of eculizumab in paroxysmal nocturnal hemoglobinuria. *Blood* 2005;106:2559-2565.

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≡ PIG-A MUTATIONS IN NORMAL HEMATOPOIESIS

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Although the molecular biology and biochemistry of PNH have been elucidated, the mechanism of clonal dominance is not completely understood in PNH. Because of its close association with acquired aplastic anemia, an autoimmune form of bone marrow failure, a 2-step model has been proposed to explain the clonal expansion in PNH. This model proposes that PIG-A mutations are common benign events in hematopoietic stem cells (Step 1) and that the PNH clone expands when normal stem cells – but not the PNH stem cell – are under immunologic attack (Step 2). In support of this theory, *PIG-A* mutant granulocytes and lymphocytes have been found in most healthy subjects at a frequency of roughly 1 in 50,000 (see **Sources for Additional Information 1-3** at the end of this program)

Hu and colleagues isolated CD34^{pos} progenitor cells from blood and mobilized peripheral blood, and analyzed the frequency of PIG-A mutant progenitor cells by assaying for colony forming cells in the presence of toxic doses of proaerolysin. Proaerolysin resistant CFC had a frequency of roughly 1 in 50,000, remarkably similar to the frequency of PIG-A mutant granulocytes found in healthy subjects. However, upon DNA sequencing of individual proaerolysin resistant CFC, a striking difference was found when PIG-A mutations from PNH patients were compared to those found in healthy controls. PIG-A mutations from PNH patients were clonal and involved all lineages including T cells. In contrast, PIG-A mutations from healthy controls were polyclonal and did not involve T cells

These data demonstrate that PIG-A mutations in PNH occur in a multipotent hematopoietic stem cell. In contrast, most, if not all, PIG-A mutations that occur in healthy subjects occur in colony forming cells that have no self-renewal capacity. These data suggest that PIG-A mutations in control subjects may be a function of normal differentiation. DNA repair is progressively attenuated during cellular differentiation, possibly leading to differentiation-dependent spontaneous mutations. Thus, PIG-A mutations found in healthy controls may have no relevance to the pathophysiology of PNH since mutations occurring in cells with little to no self-renewal capacity, CFC, are not propagated. The mechanism whereby PIG-A mutations arise in PNH stem cells and how this clone establishes itself as clonally dominant remains unclear. It is possible, as Luzzatto et al theorize (see **Sources for Additional Information 5** at the end of this program) that immune selection contributes to clonal dominance. Alternatively, it is possible that other currently unrecognized mutations confer a growth advantage to the PIG-A mutant clone.

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Learning Objectives

At the conclusion of this activity, participants should be able to:

- Discuss the importance of quantifying the size of the PNH clone.
- Describe the mechanism of action of eculizumab.
- Explain the relevance of PIG-A mutations in normal hematopoiesis.

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