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Paroxysmal Nocturnal Hemoglobinuria: Pathogenesis, Testing, and Diagnosis

Plus a Case Report and a Q&A on the Role of the Hematopathologist

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Paroxysmal Nocturnal Hemoglobinuria: Pathogenesis, Testing, and Diagnosis

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Abstract: Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal hematopoietic stem cell disorder with far-reaching multisystem effects that can lead to life-threatening consequences. The clinical features of this disease arise as a result of complement-mediated hemolysis in unprotected red cells, leukocytes, and platelets as well as the release of free hemoglobin that occurs with erythrocyte destruction. Patients may present with a variety of clinical manifestations, such as anemia, thrombosis, kidney disease, smooth muscle dystonias, abdominal pain, dyspnea, and extreme fatigue. There are 3 broad patient categories in whom the incidence of PNH is significantly greater as compared with the general population: patients with hemolysis or hemoglobinuria, patients with bone marrow failure syndromes, and patients with unexplained or unusual thrombosis. The gold standard test for PNH diagnosis consists of high-sensitivity flow cytometry performed on a peripheral blood sample. Recent advances in treatment have emphasized the importance of early diagnosis.

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal hematopoietic stem cell disorder that results in complement-mediated hemolysis arising from the affected stem cell population, which in turn produces severe and even potentially fatal clinical consequences. Until recently, supportive and symptomatic treatment was all that could be offered to these patients. As such, early diagnosis was generally not considered to be a compelling issue. However, with better understanding of the pathophysiology of PNH, the management of these patients has now progressed beyond just supportive care to a very specific targeted treatment of the underlying disease process of complement-mediated hemolysis.[1] This therapeutic advance, with the consequent improved clinical outcomes, has now made it important to recognize and diagnose these patients in an effective and timely fashion.

Disease Pathogenesis

Although PNH has been recognized for more than a century, a deeper understanding of the underlying biology of the disease has only recently become available.[2] Contributing to the confusion surrounding PNH is the name itself, which seems to suggest that the condition is limited to hemolysis and anemia. In fact, it is now known that PNH has more profound consequences. Frequent manifestations of PNH include smooth muscle dystonias, chronic kidney disease, and thrombosis.[3,4] These conditions are not solely explained by either anemia or hemolysis. Instead, research into the pathogenesis of PNH has revealed that the disease arises from chronic and uncontrolled complement activation,

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causing dysfunction in all 3 blood cell lines: red blood cells, white blood cells (both granulocytes and monocytes), and platelets.

A component of the immune system, the complement system is composed of more than 30 proteins generated in the liver and present in the blood circulation and within body tissues. Upon activation, complement proteins conduct immune activity against invading organisms or foreign materials. C3 is a key molecule in this system whose activation triggers most of the downstream effects. Owing to a labile thioester bond, a small amount of activated C3 is always present, which maintains a constant low level complement activation, called "C3 tick over," and allows for full-scale amplification when needed. One of the downstream components of the complement system is the membrane attack complex, which is designed to disrupt and destroy cell membranes. Typically, proteins on the surface of cells that inhibit complement protect cells against the membrane attack complex; however, patients with PNH lack these protective proteins on the surface of their bone marrow stem cells. The cause of this deficiency is an acquired clonal deletion of glycosylphosphatidylinositol (GPI) anchors, which typically tether certain proteins to the cell surface (Figure 1).[5,6] Specifically, the genetic abnormality has been traced to a mutation of the phosphatidylinositol glycan class A (*PIG-A*) gene. Without these complement inhibitors on the surface of the cells, the cells are subjected to complement-mediated hemolysis, which in turn produces the clinical manifestations of the disease. Since the *PIG-A* mutation occurs in bone marrow stem cells, it is imparted to all of the cell lines arising from them. Also, since it is an acquired abnormality, the involvement of bone marrow stem cells may range from a small percentage in some cases to more than 90% in others. This "percentage" is accordingly reflected in the circulating blood cells and is referred to as the *PNH clone*.

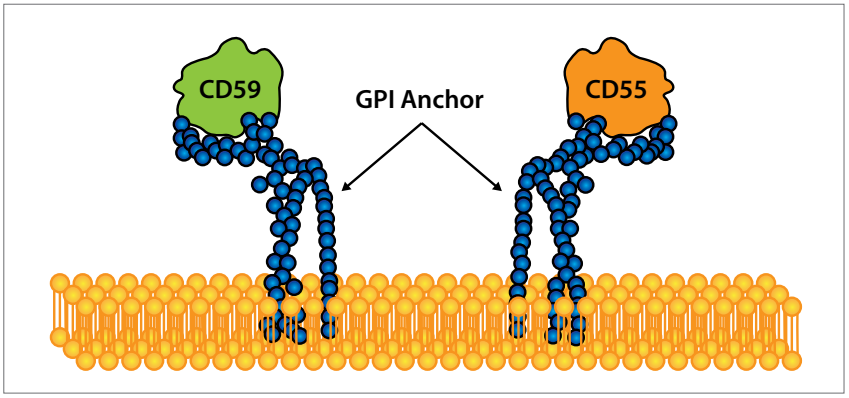

Figure 1. The cause of the deficiency in paroxysmal nocturnal hemoglobinuria cells is an acquired clonal deletion of glycosylphosphatidylinositol (GPI) anchors, which typically tether certain proteins to the cell surface. CD59 (Membrane Inhibitor of Reactive Lysis) forms a defensive shield for red blood cells against complement-mediated lysis and inhibits the assembly of the membrane attack complex. CD55 (Decay Acceleration Factor) prevents formation and augments instability of the C3 convertases, attenuating the complement cascade. Adapted from Brodsky R. Paroxysmal nocturnal hemoglobinuria. In: *Hematology—Basic Principles and Practices*. 2005:419-427.[39]

Clinical Manifestations: Morbidity and Mortality

The clinical features of this disease arise as a result of complement-mediated hemolysis in unprotected red cells, leukocytes, and platelets as well as the release of free hemoglobin that occurs with erythrocyte destruction.[1] Free hemoglobin is toxic, and it has 2 primary consequences. The first is progressive renal damage resulting from the buildup of hemoglobin deposits in the kidney, which can culminate in renal failure. Second, free hemoglobin causes depletion of nitric oxide, a compound important for maintaining smooth muscle relaxation and normal endothelial function. This depletion triggers multiple effects, including impaired regulation of smooth muscle tone, platelet hyper-reactivity, and local vasoconstriction. Among the clinical effects of nitric oxide depletion are smooth muscle spasms and ischemia resulting in dysphagia, chest pain, and abdominal pain; dyspnea; pulmonary hypertension; increased risk of thromboses; and erectile dysfunction.

In addition to the loss of red blood cells, PNH also increases leukocyte and platelet activation and aggregation. Unlike erythrocytes, these cell types are not destroyed but rather become activated upon complement-induced damage. Activation of white blood cells induces the production and release of cytokines, resulting in inflammation and debilitating fatigue.[7] Notably, this fatigue is not adequately treated with transfusion because cytokines, and not just anemia, are the primary causes.[8] Similarly, platelets injured by the complement system release procoagulant microparticles that increase the risk of thrombosis.[9]

It is apparent from the above discussion that PNH is much more than a disease of hemolysis and anemia and in fact has far-reaching multisystem effects, often with severe and even life-threatening consequences.

PNH can result in early mortality. In a study from the United Kingdom, approximately one-third of patients (35%) died within 5 years of their diagnosis despite best supportive care (Figure 2).[10] The patient cohort in this study exhibited elevated hemolysis associated with thrombosis and renal failure—2 major causes of PNH-related mortality. Historical studies show the median survival to be between 10 and 15 years from the time of diagnosis.[11,12]

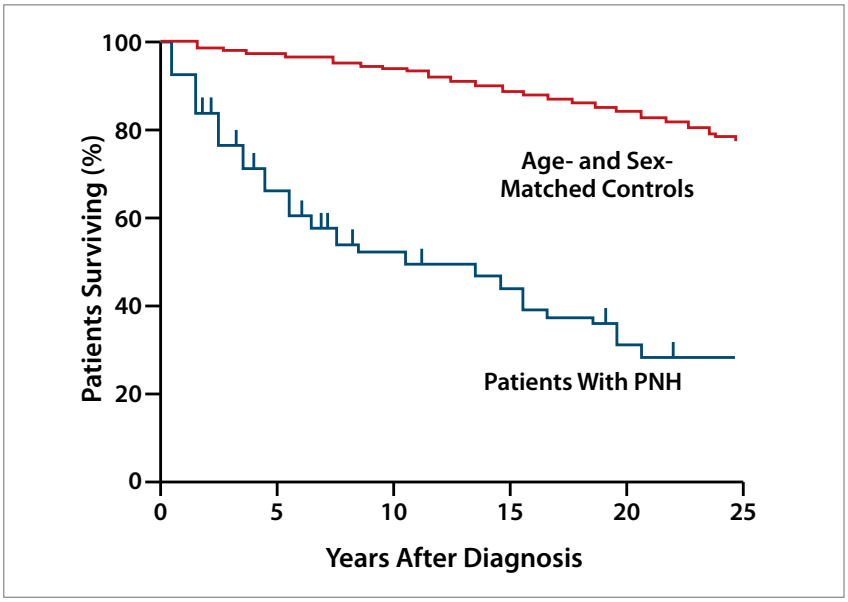

Figure 2. In a study from the United Kingdom, approximately one-third of patients (35%) died within 5 years of their diagnosis despite best supportive care. The expected survival of an age- and sex-matched control group is shown for comparison. Adapted from Hillmen P et al. *N Engl J Med.* 1995;333(19):1253-1258.[11]

In a more recent retrospective study of 460 French patients diagnosed from 1950 to 2005, the median survival had increased to 22 years.[13] The decreased mortality in this study may be attributable to the high rate of patients with aplastic anemia, who may have better survival due to immunosuppressive therapy. In addition, this study did not distinguish between hemolytic and nonhemolytic PNH patients; survival would be expected to be higher in nonhemolytic patients.

The combination of nitrous oxide depletion, complement-mediated platelet activation, and exposure of the procoagulant interior of the red cell membranes makes PNH an extremely hypercoagulable state. In fact, thrombosis is the leading cause of death among patients with PNH, and accounts for 40% to 67% of the mortality from the disease.[11,12,14-16] A history of even 1 thrombotic event is associated with a 5-fold to 10-fold higher risk of death for PNH patients.

Chronic kidney disease has been reported to occur in a majority (64%) of PNH patients,[17] with the prevalence being more than 6 times that of the general population.[17,18] One large study found that renal failure was the cause of death in 8% to 18% of these patients.[16]

Pulmonary hypertension occurs in nearly half of PNH patients and often manifests as dyspnea (66%), which is moderate to severe in 72% of patients. The severity of the dyspnea is generally independent of the patient's hemoglobin level, again pointing to pulmonary hypertension or occult pulmonary emboli as the primary cause in most cases.[19]

An analysis of 301 PNH patients from a South Korean registry evaluated the association between certain hemolytic symptoms and thrombotic risk as well as mortality.[20] A multivariate analysis showed that the odds of experiencing thrombosis were significantly higher among PNH patients with abdominal pain (odds ratio, 2.8; *P*=.006), chest pain (odds ratio, 2.7; *P*=.022), or dyspnea (odds ratio, 2.9; *P*=.003) compared with PNH patients who had no such symptoms. Elevated LDH was shown to be an independent risk factor for thrombosis. There was a 7-fold increase in mortality in patients who had a thrombotic event. In a 1996 analysis of a French PNH registry, poor survival was associated with thrombosis (relative risk, 10.2; 95% CI, 6-17; *P*<.0001).[12] Some data have suggested that thrombosis incidence and mortality in PNH is much higher in Western patients than Asian patients, in contrast to the rates of mortality from renal failure.[16] In a study comparing the clinical course of PNH in the United States and Japan, a new thrombotic event was significantly more common in Western patients vs Asian patients (31.8% vs 4.3%; *P*<.0001), and thrombosis was the cause of death in 42.1% of Western patients vs 7.9% of Asian patients (*P*=.0006).[16] The reason for the low rates among Asian patients seen in this study is not well understood. Lee and colleagues suggested that they may reflect underreporting, as data were based on physician questionnaires and not medical records.[20] Also of note, thrombotic events were seen regardless of granulocyte clone size (19%, 37%, and 44% in patients with clone sizes of <20%, 20%-50%, and >50%, respectively). Thus, even PNH patients with a relatively small clone size can be at significant risk of experiencing thrombosis and other related morbidity. Finally, a disorder of bone marrow failure such as aplastic anemia may be seen in association with PNH and may be found before or after a diagnosis of hemolytic PNH.[12,20]

Historical Treatments

Essentially all treatments that have historically been used to treat PNH are generally supportive and symptomatic in nature, and do not treat the underlying cause of the disease—complement-mediated blood cell damage and its consequences.[21] One of these palliative therapies is blood transfusion, which may be effective for attenuating anemia-related symptoms. However, its effects are only temporary, and repeated transfusions

may result in iron overload. Further, this approach does not address hemolysis and other complement-mediated effects that drive most of the severe clinical manifestations of the disease. Corticosteroids have been used in PNH patients to improve hemoglobin levels. However, they achieve only a partial reduction in hemolysis, and their significant side effect profile limits their use, especially as long-term treatment.[22] Anticoagulant therapy has been used for thrombotic events but has had a high failure rate in PNH patients.[23] In addition, complications of the disease, such as thrombocytopenia and hemorrhage, may further militate against the use of anticoagulants for PNH. Androgen therapy has been occasionally used to treat anemia in PNH patients, although this option has very limited efficacy and is difficult to use as long-term therapy, especially in female patients. Bone marrow transplantation (BMT) is currently the only curative treatment option for PNH patients. However, it has several drawbacks, foremost of which are high rates of morbidity and mortality. For example, long-term results of a study of 26 PNH patients who underwent treatment with BMT reported a transplant-related mortality rate of 42%.[24] The cumulative incidence of graft failure, a long-term complication of BMT, was 8% among these patients. Of the 15 patients who remained alive, all showed complete hematologic recovery, with no evidence of PNH. The 10-year probability of disease-free survival was 57%. The overall incidence of chronic graft-vs-host disease was 50% among 20 evaluable patients and the condition was considered extensive in 16% of patients.[24]

A modality like BMT, which appears to be associated with a significant short-term mortality risk in PNH patients, needs to be approached with very careful consideration of the above issues and should probably be reserved for patients with an associated life-threatening bone marrow failure.

With the exception of BMT, these treatments are primarily for symptoms and do not address the underlying cause of the disease—chronic complement-mediated hemolysis. Overall outcomes, therefore, have understandably been less than optimal.[11]

Diagnosis

High-Risk Groups

Despite what its name seems to suggest, PNH does not necessarily present in a dramatic fashion with sudden episodes of dark, cola-colored urine in the morning. In fact, the presentation is usually more subtle and can be missed or mistaken for some other condition unless the physician is diligent in considering the diagnosis and initiating definitive testing in the appropriate clinical settings. Certain manifestations of the disease should prompt PNH screening, especially when they occur concomitantly with anemia or cytopenias. These signs and symptoms at presentation may include abdominal pain, dyspnea, thrombosis, and extreme fatigue. Since many of these signs and symptoms are seen quite frequently in clinical practice, it would be impractical to routinely screen all patients who present with them. Thus, clinicians must focus on recognizing specific high-risk groups for testing. Guidelines from the International Clinical Cytometry Society (ICCS) and the International PNH Interest Group provide recommendations for identifying these high-risk groups (Figure 3).[21,25] There are 3 broad patient categories in whom the incidence of PNH is significantly greater as compared with the general population: patients with hemolysis or hemoglobinuria, patients with bone marrow failure syndromes, and patients with unexplained or unusual thrombosis.

Hemolysis or Hemoglobinuria. The first high-risk group includes patients with hemolysis or hemoglobinuria. PNH screening is not necessary for patients with antibody-mediated Coombs-positive hemolytic anemia. Routine PNH testing should be performed, however, in patients with Coombs-negative hemolytic anemia and in patients who have no other obvious cause for hemolysis, particularly when it is associated with abdominal pain or dyspnea. PNH testing should also be performed in patients with unexplained hemoglobinuria, either microscopic or gross. In one large study, 22.7% of patients with Coombs-negative hemolytic anemia and 18.9% of patients with hemoglobinuria were found to be positive for PNH clones.[26]

Bone Marrow Failure Syndromes. The second high-risk group consists of patients with bone marrow failure syndromes, including aplastic anemia, myelodysplastic syndrome (MDS; typically the refractory anemia subtype), and unexplained cytopenias. It is especially important to recognize this group because there is a well-established association between bone marrow failure and PNH; approximately 45% of PNH patients have bone marrow failure syndromes.[27] The association between PNH and aplastic anemia is clear, with studies reporting that 57% to 70% of patients with aplastic anemia have detectable PNH clones.[21,28,29] Aplastic anemia may be found before or after a diagnosis of hemolytic PNH.[13,20,30] In addition, PNH frequently arises following effective immunosuppressive therapy for aplastic anemia.[31] Between 20% and 50% of MDS patients have been reported to have PNH clones,[21,30] and approximately 50% of patients with other forms of bone marrow failure syndromes have such clones.[32] The value of identifying PNH clones in this group of patients is 2-fold. First, it may reveal clinically significant PNH requiring treatment to control symptoms and prevent complications. Second, the presence of a PNH clone is a marker for response to immunosuppressive therapy among patients with

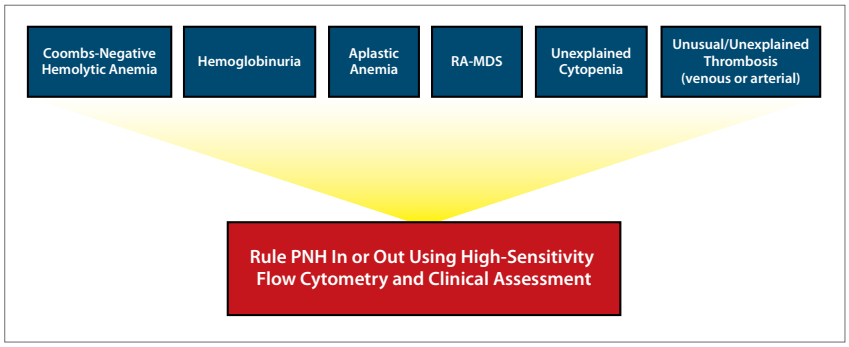

Figure 3. Guidelines from the International Clinical Cytometry Society (ICCS) and the International PNH Interest Group provide recommendations for identifying these high-risk groups. Adapted from Borowitz MJ et al. Part B. *Clin Cytometry*. 2010;78B:211-230[25] and Parker C et al. *Blood*. 2005;106(12):3699-3709.[40]

bone marrow failure syndromes, and it has been shown that both aplastic anemia and MDS patients with detectable PNH clones of any size have an improved response to such treatment as compared with those who do not have such clones.[30,33]

Although the association between PNH and bone marrow failure syndromes is well established, most estimates of their combined incidence are based on studies with small sample sizes and variable diagnostic techniques. The recently completed EXPLORE (Examination of PNH, by Level of CD59 on Red and White Blood Cells) trial was a large, multicenter study that prospectively investigated the prevalence of undiagnosed PNH in patients with bone marrow failure syndromes, including aplastic anemia, MDS, and other bone marrow failure syndromes.[34] The presence of at least 1% granulocyte PNH clones was found in 18.5% of aplastic anemia patients and 1.1% of MDS patients. Additionally, 3.6% of patients with other forms of bone marrow failure had at least 1% granulocyte PNH clones. Of note, the EXPLORE study also tested the same samples using high-sensitivity flow cytometry (which detects clones up to 0.01%, as compared to 1% with routine sensitivity), and found a higher prevalence of detectable PNH clones in all categories including aplastic anemia (70%), RA-MDS (17%), and other (55%). This finding is particularly significant when one considers the fact that detection of any size PNH clone is a biomarker for high likelihood of response to immunosuppressive therapy in these patients.[30,33] Thus, high-sensitivity flow cytometry should be the preferred method of screening. Significantly more patients diagnosed with other bone marrow failure syndromes (76.9%) had PNH clone sizes of 10% or higher compared with patients with aplastic anemia (50.4%; P=.001) or MDS (54.0%; P<.001). The median clone size for each patient subgroup was 5.1% in aplastic anemia patients, 17.6% in MDS patients, and 24.4% in other bone marrow failure syndrome patients.

Unexplained or Unusual Thrombosis. The third high-risk group consists of patients with unexplained or unusual thrombosis. Examples include thromboses in sites such as the mesenteric vein or the hepatic vein (Budd-Chiari syndrome), cerebral vein thromboses, and unexplained thromboses such as strokes, myocardial infarctions, pulmonary emboli, and deep vein thromboses. Patients who experience a thrombotic event in conjunction with another clinical feature of PNH (such as anemia, severe fatigue, pulmonary hypertension, or cytopenias suggestive of bone marrow failure) should also be considered candidates for testing. Although thrombosis is the most common cause of mortality in PNH patients and is a common complication of the disease (occurring in 40% of patients), PNH accounts for only a small proportion of cases of unexplained thromboses. In one study, 1.4% of patients with unexplained thrombosis were positive for a PNH clone.[26] Finally, another category to consider is those patients who do not respond to anticoagulation therapy, as PNH is associated with a severely hypercoagulable state resulting in a high rate of failure of these agents.

It is important to realize that since PNH is a relatively rare disease, many tests will in fact come back negative, which could lead to "testing fatigue." It is therefore very useful for clinical practices to have an algorithm in place that identifies these high-risk patient populations on a regular and systematic basis to help ensure that PNH diagnoses are not missed. Such an algorithm typically takes the form of flagging paper or electronic records based on International Classification of Diseases (ICD) codes for each of the high-risk categories described above.

Testing

Once the high-risk patient has been identified, definitive testing for PNH should be performed. Unlike previous generations of tests—such as Ham's (acid hemolysis) test and the sucrose hemolysis test—the current test for PNH is quite simple. The gold standard test for PNH diagnosis consists of high-sensitivity flow cytometry performed on a peripheral blood sample.[25] The flow cytometry assay detects the binding of monoclonal antibodies directed against cell-bound complement regulators. PNH flow cytometry test results should include: (1) the clone size for each cell lineage; (2) the proportion of type I, II, and III cells in PNH-positive cases (described below); and (3) the sensitivity level used (high sensitivity, or 0.01%, is considered optimal). These results should be compared with all prior

flow cytometry results to monitor clonal expansion.[25] Flow cytometry should be performed in high-quality pathology laboratories that emphasize best practices and have staff trained and experienced in hematopathology.

The ICCS has provided several key recommendations for conducting flow cytometry analysis in the diagnosis of PNH. Chief among these recommendations is that more than 1 blood cell line should be analyzed to increase the confidence of the interpretation and diagnosis. Because affected red blood cells undergo hemolysis and destruction, a false-negative result can occur if all of the abnormal erythrocytes have been destroyed. Further, reduction in the number of PNH-positive red blood cells resulting from hemolysis and dilution following red blood cell infusion therapy can greatly underestimate the true PNH clone size using this cell type. Thus, both red and white blood cells are typically included for flow cytometry analysis. Although monocytes can be useful in the diagnosis of PNH, their population is generally smaller in the peripheral blood, limiting the ability to identify very small clone sizes and making granulocytes a more useful population for determination of PNH clone size. Importantly, levels of red blood cells that are far lower than those of white blood cells may be indicative of the level of hemolysis.

The ICCS has also provided recommendations regarding the optimal cell surface proteins to assess in flow cytometry. For red blood cells, CD59 is recommended; normal red blood cells are associated with robust CD59 expression, whereas red blood cells with the PNH genetic abnormality show either partial or complete loss of expression. The different populations of red blood cells are referred to as type I (normal), type II (intermediate complement sensitivity), and type III (most abnormal PNH type). Flow cytometry with at least 2 reagents is recommended for detection of granulocytes, owing to the potentially low

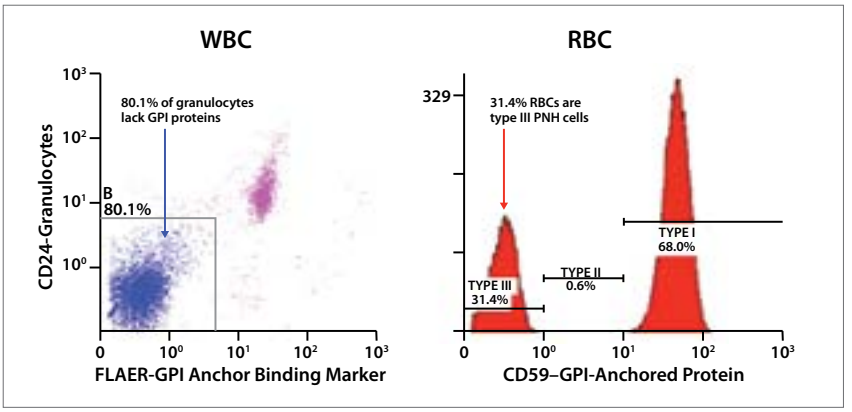

Figure 4. The results of a flow cytometry test evaluating the granulocytes and RBCs in a known PNH patient are depicted. FLAER, fluorescently-labeled aerolysin; GPI, glycosylphosphatidylinositol; PNH, paroxysmal nocturnal hemoglobinuria; RBC, red blood cell; WBC, white blood cell. Figure courtesy of Alexion.

number of cells that may be available in some patients. The results of a flow cytometry test evaluating the granulocytes and red cells in a known PNH patient are depicted in Figure 4. Fluorescently-labeled aerolysin (FLAER), a bacterial protein that strongly and specifically binds to the GPI anchor on the surface of normal white blood cells, is recommended for detection of PNH granulocytes which lack the anchor and therefore do not bind to this reagent. Other targets on white blood cells include CD24, CD66b, and CD16. Note that CD59 is not recommended for assessing white blood cells because the use of FLAER has been shown to be more sensitive for these cells than flow cytometric assays that measure CD59.[35,36] The most common test for monitoring hemolysis in PNH patients is evaluation of serum lactate dehydrogenase (LDH) enzymatic activity.[21] LDH levels are frequently elevated in PNH patients, and in cases of severe exacerbations, levels may be greater than 20 times the upper limit of normal.[2] An LDH greater than 1.5-fold above normal is a risk factor for PNH consequences such as thromboembolism and abdominal pain.[20]

There are several other laboratory tests commonly performed in PNH patients, many of which are focused on evaluating the presence and/or progression of complications arising from the disease. For example, measurement of proteinuria and serum creatinine levels is appropriate in these patients, given their increased risk of chronic renal disease. Measurement of D-dimers, fibrin degradation products that form as a result of fibrinolysis or plasmin-mediated clot degradation, can be used to assess active coagulation and fibrinolysis. D-dimer testing can be an important tool in the evaluation and management of thrombosis events.[37]

Role of Testing in Follow-Up

Once a diagnosis of PNH is made, guidelines recommend subsequent routine follow-up testing with flow cytometry, repeated every 3 to 6 months initially and then annually thereafter.[25] Repeated testing is recommended because PNH arises from an acquired mutation, and, therefore, the clone size can vary over time.[32,38] Generally, the clone size either remains stable (in approximately 20%-50% of patients over a 5-year period) or increases (in approximately 20% of patients).[32] In approximately 10% to 15% of cases, however, the clone decreases in size or even disappears with time. This routine follow-up testing is recommended regardless of the therapeutic strategy administered. For example, a patient with a very small clone who is being followed with observation may need

to be treated if the clone expands over time. Conversely, a patient on treatment may be able to discontinue it if the clone is no longer detectable on long-term follow-up.

Ultimately, the goal of treatment for these patients is to manage the chronic complement activity and, as a consequence, ameliorate symptoms, improve quality of life, and prevent the severe disease-related complications. Optimal disease control requires early intervention and treatment addressing the underlying hemolysis.

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Paroxysmal Nocturnal Hemoglobinuria: Case Study Discussion

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A 68-year-old woman with a history of type 2 diabetes and hypertension was referred to the hematology clinic for progressive fatigue, bruising over extremities, and pancytopenia. Her platelet count was 32,000/µL, her hemoglobin was 10 g/dL, and her white blood count (WBC) was 3500/µL. Her platelets subsequently deteriorated further such that she started to need regular transfusions. An extensive workup, including measurement of vitamin B_{12} and folate levels; serum protein electrophoresis; thyroid-stimulating hormone testing; serology for human immunodeficiency virus; and testing for hepatitis, *Cytomegalovirus*, Epstein-Barr virus, and parvovirus B19, was nondiagnostic. A Coombs test was also negative, and abdominal ultrasound did not reveal any splenomegaly. Coagulation tests were normal. A lactate dehydrogenase (LDH) level was slightly above the laboratory's normal range at 650 mg/dL, but was considered nonspecific and insignificant at the time. A bone marrow biopsy was performed, which showed erythroid hyperplasia with reduction of granulocytic precursors and marked reduction of megakaryocytes. There were no definitive morphologic features of myelodysplasia, and cytogenetic studies were also normal.

The patient was treated with supportive measures, including platelet and occasional red cell transfusions, but became progressively more symptomatic over the next 6 months with worsening fatigue and more pronounced symptoms of dyspnea on exertion as well as episodes of abdominal pain. A subsequent workup failed to reveal any cardiac or pulmonary cause for her symptoms, and abdominal imaging was also unremarkable.

At this time, her case was carefully reviewed, and the possibility of paroxysmal nocturnal hemoglobinuria (PNH) was considered. A peripheral blood sample was sent for flow cytometry to assess for PNH. The test was done in accordance with guidelines from the International Clinical Cytometry Society (ICCS) and showed a detectable PNH clone in 2.7% of her granulocytes and 1.1% of her red cells. Given the severity of her condition, she was started on specific anti-complement therapy, which resulted in dramatic improvement of her symptoms over the next 4 weeks. However, she continued to require platelet transfusions owing to her bone marrow failure. Since the presence of a PNH clone in the setting of bone marrow failure is a marker for a high likelihood of response to immunosuppressive therapy, she was given a trial of cyclosporine. Her platelet count subsequently improved to more than 80,000/µL over the next several weeks, and she has remained transfusion-independent since then.

This case exemplifies many of the key points discussed in the previous article. In particular, it highlights the need to maintain an elevated index of suspicion for the possibility of PNH in the high-risk categories of hemolysis, thrombosis, and bone marrow failure, particularly when other clinical signs and symptoms of the disease are present. This patient had severe fatigue that was out of proportion to the degree of anemia, and she also had episodes of abdominal pain, both of which are potential manifestations of PNH. Had this been considered during her initial workup, along with the fact that she was in one of the high-risk groups (bone marrow failure), she could have been diagnosed and treated several months earlier, potentially avoiding many transfusions, not to mention the quality-of-life consequences. Also, this patient was fortunate in that the diagnostic delay did not result in any of the potentially catastrophic thromboembolic complications of PNH. Another interesting aspect of this case is the severity of the patient's symptoms with what would be considered a relatively small PNH clone. The dramatic improvement after complement-directed therapy rules out other explanations. It highlights the fact that many of the symptoms of the disease may not be entirely due to hemolysis but may instead be caused by complement-mediated damage to other unprotected cell lines, such as granulocytes or platelets, which may be able to produce severe manifestations including cytokine-driven fatigue and platelet activation–driven thrombosis even with relatively small populations of cells. This theory, however, has not been well studied.

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Paroxysmal Nocturnal Hemoglobinuria: The Role of the Hematopathologist

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Vivek R. Sharma, MD (VS) We know that paroxysmal nocturnal hemoglobinuria (PNH) is a disease that is uncommon. It is extremely severe, however, and we now have more effective therapies. So it has become particularly important for clinicians to diagnose it in a timely and effective manner. What, in your opinion, should hematopathologists know about this disease?

Sameer S. Talwalkar, MD (ST) Many hematopathologists are aware of the disease and how clinically important it is. The reason I believe it should be given more emphasis now is because there is an effective treatment. The onus is on the laboratories that are performing the PNH assay to provide quality results in a timely fashion. PNH is an ultra-rare disease, but it is life-threatening. Patients must receive an accurate and prompt diagnosis to avoid compromising treatment and outcome.

VS That is a very significant point. The role of hematopathologists is crucial because the final diagnosis is made based on the tests they run. How can a hematopathologist ensure that the tests are administered properly and the results are reported in a clear and appropriate format? Are there any specific guidelines that hematopathologists should follow?

ST In the past few years, the International Clinical Cytometry Society (ICCS) has published guidelines covering who should be tested, how testing should be performed, and how the results should be reported. As a hematopathologist, I routinely use these guidelines in day-to-day practice. A hematopathologist who is well trained in flow cytometry should be able to make the diagnosis of PNH.

Even laboratories with a basic 4-color flow cytometer can set up a PNH assay to provide accurate and timely results to physicians. Having said that, there are certain points to stress in terms of the quality and validation of this assay. I recommend running several negative samples and comparing these to a few known positives to see differences in staining and scatter properties of normal cells compared with PNH cells. From a clinical perspective, it is also very important to examine at least 2 different cell populations, ideally red cells and granulocytes.

VS Why is it necessary to examine 2 different cell populations?

ST First of all, the red cells in a patient who has untreated PNH undergo hemolysis, and therefore one may not get an accurate red cell clone size or may even get a false-negative result if looking at red cells alone. The second issue concerning red blood cells is that laboratories that do not use the CD235a (Glycophorin A) antibody for measurement might obtain false-positive results as a result of not gating on a pure red cell population. The addition of granulocytes therefore increases not only sensitivity but also specificity of this assay by reducing the false positives.

VS I have worked with some laboratories that have sent reports based on testing of only the red cells. From what you are saying, that approach is suboptimal and contradicted by ICCS guidelines.

ST I would not recommend it.

VS In terms of the sample, is it possible for clinicians to send bone marrow to test for PNH, or is peripheral blood preferred?

ST The recent ICCS guidelines recommend the use of peripheral blood. In my experience, bone marrow samples can be used in the research setting, but not in the clinical laboratory. Often these specimens have immature myeloid cells, which lack uniform expression of glycosylphosphatidylinositol (GPI)-anchored molecules or a high level of expression of these molecules. The analysis will be flawed if it is based on immature cells that do not express GPI molecules uniformly.

Another major disadvantage of using bone marrow samples involves patients with myelodysplastic syndrome, who have dysplasia in their myeloid lineage. It is often difficult to interpret PNH test results in these patients when they have a small PNH clone and compromised expression of certain GPI molecules, particularly CD16. In these patients, it may not be possible to obtain an accurate analysis with a bone marrow specimen.

VS Those are great points that likely underlie the recommendations from the ICCS. There is a good reason for everything in those guidelines.

There have been occasions when physicians have asked me questions about how to interpret the test report.

These reports can refer to different clones—type I, type II, type III. They can discuss red cells, granulocytes, and monocytes. Physicians who are less familiar with the details of PNH pathology and flow cytometry may sometimes wonder what the report is exactly saying. What are your thoughts on this issue? How can hematopathologists ensure they are providing a report that is clear, complete, and easy to understand?

ST It is very important for laboratories to communicate well in the report. Laboratories that are just starting to validate this assay or want to improve their approach can refer to the ICCS guidelines. The guidelines provide recommendations regarding how the results should be reported. My suggestion is that the results should be as concise and clear as possible. Typically, using terminology such as *positive for PNH* or *negative for PNH* is not recommended. Rather, our reports, similar to those of laboratories that follow ICCS guidelines, provide an interpretation as to whether a PNH clone is present or absent. It is also a good practice to give the individual clone size for each cell lineage tested so that the oncologist or hematologist can use these numbers to follow a patient's disease once treatment is initiated. For red cells, it is necessary to provide type I, type II, and type III clone sizes. Type I are the normal (non-PNH) red cells, whereas type II and type III are PNH red cells with partial and complete deficiency of GPI-linked molecules, respectively. With granulocytes, the clinical significance of the type II cells is uncertain. Therefore most laboratories, including ours, report only the type III cells as a positive PNH clone.

When the laboratory evaluates follow-up samples, the report should mention the previous clone size and highlight any changes in size that may be seen. This information might alert the clinician to the need for a change in treatment strategy.

Lastly, reporting of small clone(s) is a bit challenging, especially without a good clinical history, since it can be difficult to interpret what a small clone really means clinically. When small clones are found, it is important to document them in the pathology report and make recommendations in terms of patient follow-up.

VS Those are all great points. As you said, the key is for the laboratory to break down the technical jargon so that the results are easily interpretable by clinicians who may not be familiar with the nomenclature associated with flow cytometry. Reports that include interpretation can be very helpful and allow clinicians to more easily apply the results in the clinical context. It is also important, as you mentioned, for the report to include comments about small clones because the relevance of a small clone can be determined only over time.

ST High-sensitivity analysis can detect small clones, up to 0.01%, especially in the red cells. For laboratories that perform high-sensitivity PNH testing, it is very important to avoid false-positive results by repeating tests that are positive or having another technician perform the test, for reproducibility.

VS High-sensitivity testing simply means that more cells are counted, correct?

ST That is correct. One acquires more events so that it is possible to identify smaller clone sizes, typically as low as 0.01%.

VS It seems likely that most laboratories could set up a protocol for high-sensitivity analysis. It is a matter of taking a little extra time to count more cells.

ST Correct. The critical part of high-sensitivity analysis is to run a large number of normal samples and to acquire as many events as possible so as to facilitate differentiation of a small clone from non-specific background that may result from cell debris due to unwashed and/or unlysed cells or incomplete antibody binding to the cells. It is also very important to properly stain the cells and calibrate the instrument(s).

VS As we have discussed, the involvement of the hematopathologist is critical to the appropriate detection, diagnosis, and classification of PNH patients. I have one final question for you, Dr Talwalkar. How can the clinical hematologist/oncologist and the hematopathologist best collaborate in the comprehensive management of patients with PNH in terms of diagnosis, treatment, and follow-up?

ST It is important for the hematopathologist to understand how critical it is to provide accurate and timely test results so that the patient can be started on treatment right away. To answer your question, it should be a team effort. Communication is critical. Ideally, knowing the referring oncologist/hematologist is useful for better communication and follow-up.

It might be useful to have a "PNH team," whereby the nurse practitioner, the hematopathologist, and the hematologist/oncologist work together to manage the group of PNH patients in their practice to ensure that follow-up is effective and accurate. A good hematopathologist should be able to guide the clinical team in this effort.

VS I agree that, if possible, having a PNH team is very helpful. Once the diagnosis is made, it should be communicated clearly and expeditiously to the clinician. Since PNH is a disease that can have fluctuations, it is important to have continued monitoring and communication between the clinicians and the hematopathologist.

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