Review:
Laboratory Diagnosis of Paroxysmal Nocturnal Hemoglobinuria

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Abstract. Paroxysmal nocturnal hemoglobinuria (PNH) is an uncommon acquired stem cell disorder associated with periodic hemolytic events. This benign clonal disease is caused by abnormalities of the X-linked phosphatidylinositol glycan class A (PIGA) gene and is associated with cytopenias and thrombosis. Although the trilineage of bone marrow elements is affected, involvement of the red blood cell (RBC) line was recognized first due to its abnormal sensitivity to complement-mediated intravascular hemolysis. Totally or partially deficient blood cell membrane proteins include decay accelerating factor (DAF, CD55), membrane inhibitor of reactive lysis (MIRL, CD59), and other proteins attached to the glycoprophosphatidylinositol (GPI) spine. Stem cell transplantation can be curative in PNH. Diverse laboratory abnormalities observed in PNH include bone marrow hyper- and hypoplasia, hematologic cytopenias, micro- and macrocytosis, decreased leukocyte alkaline phosphatase (LAP), hemoglobin- and hemosiderinuria, as well as associated iron deficiency. The more definitive laboratory tests comprise older biochemical and newer flow cytometric (FCM) procedures. The former group includes the sucrose hemolysis test for screening and Ham's acid hemolysis test for confirmation; the latter group includes FCM analyses of CD55 and CD59, which have recently replaced Ham's test, and FCM quantification of specific GPI-anchor binding using fluorescent-labeled inactive toxin aerolysin (FLAER). FLAER is more sensitive than FCM quantification of antibody-binding to CD59 for PNH diagnosis. (received 1 May 2003; accepted 9 June 2003)

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Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) has engendered much attention during the past 140 years. This disease is characterized by hemolysis, anemia, thrombosis, and cytopenia. PNH is an acquired, clonal abnormality that affects the trilineage of hematologic cells. Although PNH is a non-malignant condition, it has a prolonged clinical course that can be associated with aplastic anemia and, ultimately, acute leukemia. In the past 2 decades the total or partial lack of anchoring glycoproteins associated with this condition has been identified and the gene responsible for synthesis of the blood cell membrane proteoglycan anchor has been located. Laboratory studies are indispensable to the diagnosis and monitoring of this disorder.

Historical Perspective

The syndrome of PNH was first recognized in the second half of the nineteenth century. Paul Strübing [1] differentiated PNH from both paroxysmal cold hemoglobinuria and march hemoglobinuria (Table 1). He concluded that the hemolysis was intravascular and did not occur in the urine or kidney. He theorized that PNH red blood cells (RBCs) were especially sensitive to sleep-induced acidosis. The name, paroxysmal nocturnal hemoglobinuria, was
given to this disease by a Dutch physician, Ennekin, in 1928 [1]. In 1911 another Dutchman, van den Bergh, showed that in PNH the RBCs became lysed upon exposure to CO$_2$ in either the patient’s serum or in normal serum, indicating that the PNH RBCs were abnormal. He also demonstrated that hemolysis was dependent upon a heat-labile serum factor [1].

Between 1937 and 1939, Ham showed that bicarbonate diminished hemolysis in PNH and that an acidifying agent, ammonium chloride, increased PNH hemolysis [1,2], thereby implicating acidosis as a cause of hemolysis. Ham also concluded that PNH RBCs were abnormal and that the mode of hemolysis in PNH was not antigen-antibody reliant.

In 1944, Dacie linked PNH to bone marrow aplasia in Fanconi’s anemia [3]. In 1953, Crosby noted the association of PNH with thrombosis [1]. Pillemer, who elucidated the alternative complement pathway in 1954, believed that properdin was responsible for PNH-mediated hemolysis [1].

During the 1960s Dacie and Rosse, using the complement lysis sensitivity test, identified phenotypic heterogeneity, or mosaicism, among PNH RBCs [3]. Later, Rosse identified 3 PNH RBC phenotypes: I, II, and III, that exhibit normal, moderate, and severe complement sensitivity, respectively. In 1969, Aster and Enright showed that PNH platelets and neutrophils were both inordinately susceptible to complement lysis. If the undue vulnerability of PNH RBCs to complement is also considered, then Aster and Enright’s discovery established the stem cell origin of PNH [1]. Experiments in the 1970s and 1980s showed that C3 binding was increased on PNH cells due to lack of the decay activating factor protein (DAF, CD55), an inhibitor of C3 convertase. In 1989, the membrane inhibitor of reactive lysis protein (MIRL, CD59), which inhibits the membrane attack complex (MAC), was isolated [1,4] and shown to be deficient in PNH RBCs.

Over a half-century ago, leukocyte alkaline phosphatase (LAP) was noted to be decreased in PNH; later, diminished acetylcholinesterase was found in PNH RBCs and decreased 5’-nucleotidase was demonstrated in PNH lymphocytes. These enzymes were shown to be attached to the cell membrane via phosphatidylinositol. The multiple proteins that are lacking in PNH are anchored to the cell membrane by glycosylphosphatidylinositol (GPI). In the past 20 years, the gene for MIRL was identified by Kinoshita (phosphatidylinositol glycan class A, PIG-A). PNH-related mutations that affect this X-linked gene are somatic (non-germ line); females are as frequently afflicted by PNH as males, since only one X-chromosome is active in the somatic line due to lyonization [1,4].

Thus, research over the past 120-140 years has identified the causation of PNH as an antibody-independent, complement-mediated hemolysis due to the lack of GPI-anchored complement inhibitors caused by somatic mutation of the X-linked PIG-A gene (Table 1).
Laboratory Tests Relevant to PNH

As outlined in Table 2, preliminary laboratory tests and studies include a complete blood count (CBC), stained blood smear, and bone marrow examination. LAP and serum iron studies are also relevant. Reticulocytosis may be present, however, it may also be reduced relative to the level of anemia, possibly reflecting bone marrow failure. Finally, random urine specimens can be examined for hemosiderin by Prussian blue staining [5].

For many years, Ham’s test [5,6] has been the standard way to identify the PNH clone among RBCs. Only one other disease is associated with a positive Ham’s test: hereditary erythroid multinuclearity with positive acidified serum (HEMPAS), or congenital dyserythropoietic anemia (CDA) type II, which can be readily differentiated from PNH by the medical history, bone marrow aspirate morphology, and a negative sucrose hemolysis test [6-8]. Hence, Ham’s test is highly specific for PNH.

The Ham’s test is based on the tenet that complement will attach to RBCs at somewhat acid pH and that PNH RBCs are sensitive to complement fixation. Whole defibrinated blood collected in heparin is utilized. Cells from the patient and controls are tested for hemolysis with (a) unmodified serum, (b) acidified serum (pH 6.8), (c) heat-inactivated serum (55°C for 3 min.), and (d) heated serum with guinea pig complement. Positivity should be encountered with: (1) PNH cells only and (2) acidified serum; (3) positivity is undone by heating (destroying complement), and (4) is not reestablished with guinea pig complement. This test can be modified to determine complement lysis sensitivity, to distinguish PNH type II from PNH type III RBCs [9].

The sucrose lysis test [5,10-11] has been the standard screening test for PNH. Low ionic strength isotonic sucrose causes serum globulin aggregates to fix complement on the RBC surface. Consequently, a scant amount of serum added to this solution will lyse PNH RBCs preferentially to normal RBCs. Heparinized, defibrinated whole blood is employed. A small amount of type-specific serum is added to a 10% (w/v) sucrose solution with washed patient or control erythrocytes and the mixture is incubated (1 hr, 25°C). Greater than 5% hemolysis is considered positive for PNH. The sucrose hemolysis test can also be used to determine complement lysis sensitivity [11].

Flow cytometry (FCM) has recently replaced Ham’s test as the definitive test for PNH. Usually, CD55 and CD59 are both measured by FCM; depressed levels of both of these glycoproteins are consistent with PNH. CD59 is more prevalent in the membrane than CD 55, therefore, PNH type I, II, or III cells are more easily quantified by measurement of CD59 than CD55. FCM identification of small PNH clones (<5.0%) shows the greater sensitivity of FCM, relative to Ham’s test.

Clone size is more accurately enumerated with white blood cells (WBCs), because the WBC half-life is normal in PNH, whereas the RBC half-life is shortened, especially for PNH type III RBCs, due to hemolysis. Furthermore, PNH RBCs, as opposed
to WBCs, are diluted by transfusion, which additionally diminishes the value of RBCs for evaluation of clonal size. Technically, however, determination of CD55 and CD59 is more convenient with RBCs than WBCs [5].

For FCM PNH granulocyte studies, antibodies to 2 GPI-linked antigens (CD55/CD16, CD59/CD16) and another antibody to non-GPI-linked antigens (CD15/CD33), are recommended [9]. The clinical utility of clonal monitoring for PNH is under investigation [9]. Dunn et al [12] correlated Ham's test positivity with FCM quantification of GPI-anchored protein deficient erythrocytes in PNH and found that Ham's test identified FCM positive RBCs less than half of the time, illustrating the greater sensitivity of FCM in PNH [12].

Piedras and Lopez-Karpovitch [13] showed PNH FCM clonal estimation was improved by using granulocytes and monocytes, instead of erythrocytes and lymphocytes. Richards, Rawstron, and Hillmen [14] advocated the use of both CD55 and CD59 because rare patients with certain heritable conditions can lack only one or the other of these antigens, whereas PNH cells lack both due to the defective GPI-anchor.

Some hypoplastic anemia patients are too granulocytopenic for useful FCM PNH studies [14]. Richards et al [14] recommend a non-GPI-affixed granulocyte marker (CD15/CD33) and right-angle light scatter for PNH gating. Hernandez-Campo et al [15] recommend RBC lysis prior to measuring WBC CD59 and CD55; they also developed a non-lyse, non-wash method for quantification of CD59 and CD55 on WBCs, RBCs, and platelets.

Hall and Rosse [16] studied 54 PNH patients and found CD59 superior to CD55 for RBC
populations. Hsi [17] evaluated a control group and a small group of PNH patients with RedQuant (Fig. 1) and CellQuant (Beckman-Coulter Corp, Hialeah FL) FCM kits and found both kits to be satisfactory for PNH diagnosis. However, CellQuant granulocyte CD59 had an elevated false positive rate [17].

Brodsky et al [18] identified a GPI-binding toxin aerolysin, which can be used to differentiate PNH cells. Inactivated aerolysin (fluorescent proaerolysin variant, FLAER) binds to the GPI-anchor more specifically and sensitively than anti-CD59 [19]. Consequently, FLAER detects granulocytic PNH clones as low as 0.5%. Furthermore, FLAER GPI-anchor specificity precludes the need for a second marker (ie, both CD55 and CD59), rendering FLAER investigation less complex than CD membrane marker analysis in PNH [19].

Richards and Hillmen [20] believe that agranular neutrophils lessen the utility of forward angle light scatter/side-scatter (SSC) granulocyte gating for PNH and recommend the substitution of lineage specific markers (CD15/CD33) and SSC in this instance. They also assert that neither PNH platelet analysis nor clonal monitoring is clinically useful [20]. Pakdesuwan et al [21] believe that reticulocyte FCM is superior to erythrocyte FCM for PNH clonal monitoring and diagnosis. Oelschlaegel et al [22] confirmed the reliability of RedQuant and CellQuant for PNH and stressed the importance of performing both tests for PNH cells in myelodysplasia, because the PNH clone can be absent from either the erythroid or granulocyte lineages. These reports indicate that FCM is the best non-research test for PNH diagnosis and monitoring.

Although groups of antigen negative cells are suitable for FCM, it would be advantageous to develop monoclonal antibodies to PNH neoantigens for identification of these cells by positive attributes, as opposed to CD55/CD59/FLAER absence. Thus, confusion about CD55/59/FLAER presence or absence would be eased by conventional documentation of neoantigen positivity in PNH.

The somatic mutations of the PIG-A gene are frequent, varied, and seldom duplicated [3]; about $2/3$ are insertions and deletions [9]. Kinoshita et al [23,24] identified this gene, located toward the short end of the X-chromosome (Xp22.1). By 2000, 174 mutations in this gene, causing either complete, or partial deficiencies of CD55 or CD59, had been reported [24]. About $3/4$ of the mutations lead to total functional inactivation of the PIG-A gene product, while the remaining $1/4$ have residual activity, thus explaining the difference between PNH III and PNH II cells with complete or incomplete deficiency of GPI-anchored proteins, respectively [24]. PIG-A gene mutational analysis is decisive; however, currently, these methods are investigative tools [5,25-26]. Hybridization array technology could possibly be used clinically to screen for PIG-A mutations.

In conclusion, PNH is a beguiling disease that is caused by somatically mutable “hot spots” within the X-linked PIG-A gene. This non-neoplastic, clonal, stem cell disorder is best defined by FCM, which has recently superseded Ham's test, which for many decades was the dominant diagnostic test for PNH.

References
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