Paroxysmal nocturnal hemoglobinuria (PNH) is intensely studied due to its association with bone marrow aplasia and its unique antibody-independent, complement-mediated, immunologic pathogenesis. Median survival can be 15-20 years, and the natural history is very variable. This uncommon condition (U.S. incidence about 1/1,000,000) involving a clonal acquired X-linked somatic mutation of the phosphatidylinositol glycan class A (PIGA) gene, results in a partial, or total, deficiency of the glycophosphatidylinositol (GPI)-anchoring membrane proteins. The lack of these GPI-anchor membrane proteins, some of which regulate complement activity, can lead to an increased susceptibility to complement-mediated cellular destruction.

The past 150 years of research on PNH have uncovered much information; however, in relation to the present-day laboratory diagnosis of PNH, as might be expected, the past 75 years are more relevant. Paroxysmal nocturnal hemoglobinuria is an unusual condition that can manifest itself in a myriad of ways, including hemolytic anemia, which is antibody-independent, yet immunological, due to complement-mediation. The history of advances in the laboratory diagnosis of this condition can roughly be divided into 2 parts: 1) complement-mediated RBC lytic assays, beginning more than 70 years ago and 2) starting about 2 decades ago, flow cytometric (FCM) assays employing monoclonal antibodies (mAbs; primarily CD 55 and CD 59; Table 1) and fluorescent-labeled inactive toxin aerolysin (FLAER). The history of the laboratory diagnosis of PNH has recently been reviewed.
6.5 resulted in the lysis of PNH RBCs. He presciently concluded from his experimental data that the PNH defect that resided in the RBC was possibly linked to complement, and that there was no evidence of antibody involvement. Ham’s test for acid hemolysis (HT) resulted from these studies. This test is highly specific for PNH, the only cross-reacting process that is hereditary erythroid multinuclearity with positive acidified serum (HEMPAS; congenital dyserythropoietic anemia [CDA] Type II). Here, the reactive HEMPAS RBCs can be lysed by control, as opposed to patient, serum due to a reactive immunoglobulin M (IgM) antibody. Ham’s test relies on the principle that complement attaches to RBCs at a moderately acidic pH. Later, in the 1960s, the sucrose hemolysis test (SLT) was devised. This test is a superior screen for PNH. The SLT uses the principle that low ionic strength isotonie sucrose causes serum globulin aggregates to fix complement on the surface of RBCs. For many years these 2 techniques dominated studies of PNH. A more detailed description of these tests has recently been published. Expert opinion suggests that these venerable tests are now both obsolete and outmoded.

In the 1950s, work by Pillemer implicated the alternative (properdin) pathway in PNH RBC lysis. Currently, it is believed that PNH cells can be lysed by each of the 3 complement pathways: classical, alternative, and lectin. Another complement-related advance, achieved in the next decade, is associated with the complement lysis sensitivity (CLS) test involving incubation of a sensitizing cold agglutinin with varying serum dilutions as a complement source, thus comparing the sensitivity of both normal and PNH cells to lysis. These studies by Dacie and Rosse revealed a mosaic phenotype (properdin) RBC population of PNH-type II and type III cells. For completeness, it is also necessary to note the urine test for hemoglobin, an ancillary, but classic, laboratory technique relevant to this condition.

Flow Cytometric Monoclonal Antibody Era

Flow cytometric studies of PNH began approximately 20 years ago. The predominant mAbs employed are usually directed at CD 55 (decay accelerating factor [DAF]) and CD 59 (membrane inhibitor of reactive lysis [MIRL]); CD 59 is superior to CD 55 for RBCs (Table 1). Because rare disorders are associated with the absence of single GPI proteins, PNH FCM employing mAbs must identify more than 1 abnormal antigen. Also, more than 1 cell line should be evaluated. Hsi, using a small group of PNH patients, found both CellQuart and RedQuart (Beckman-Coulter, Brea, CA) adequate for discrimination of the PNH clone. Thomason and colleagues recommend the addition of CD 14 (monocytes) and CD 16 (granulocytes), which are routine FCM markers, to CD 55 and CD 59, and to search for abnormal expression of CD 14 and CD 16 for increased PNH sensitivity. Similarly, Hernandez-Campo and colleagues recommend CD 16/CD 24/CD 55/CD 59/CD 66b/CD 157 as a neutrophil panel; CD 14/CD 55/CD 157 for monocytes; CD 24/CD 48/CD 52/CD 55 for B cells; CD 48/CD 52/CD 55 for CD 4 (+) T cells; CD 55/CD 59 for eosinophils; and CD 48/CD 55 for CD 8 (+) T cells. If limited to 1 marker: CD 48 for CD 56 (low) NK cells; CD 55 for BOCA 3(-) dendritic cells plus CD 56 (high) NK cells; and CD 59 for RBCs. Dworacki and colleagues reaffirmed the value of CD 55 and CD 59 for PNH FCM and also mentioned the use of reticulocyte-gating for PNH as superior to erythrocyte gating because 1) the reticulocyte count can be elevated in PNH post hemolysis; 2) the reticulocyte fraction can be enriched by non-hemolyzed PNH RBCs; and 3) the PNH reticulocytes can be more resistant to hemolysis than PNH erythrocytes. Olteanu and colleagues identified the utility of the CD16/CD 55/CD 14/CD 45 panel for PNH bone marrow monocytes and granulocytes; CD 59 had a lower mean fluorescence intensity (MFI) for monocytes than granulocytes, making it a less valuable marker for differentiating PNH monocytes, as opposed to PNH granulocytes. The PNH granulocyte/monocyte clones were larger than erythrocyte/lymphocyte clones, reconfirming the enhanced value of PNH granulocyte clonal measurement, as opposed to PNH erythroid clonal sizing, which is affected by hemolysis and transfusion.

They confirmed the value of measuring the WBC (granulocytes, monocytes), as opposed to the erythroid, PNH clone, when percent negativity is considered. Olteanu and Xu have recently reviewed the FCM diagnosis of PNH by mAbs. Gupta and colleagues compared 4 tests for PNH: FCM, sephacryl-GCT (gel card test) for RBCs, using mAbs to CD55 and CD 59 (St. Morat, Switzerland; Dia-Med ID), the SLT, and the HT. Of 50 patients evaluated, a PNH clone was found in 28% by FCM, 26% by sephacryl-GCT, and 20% each by HT and SLT, respectively. When compared to FCM, the GCT and the lytic tests had 92.8% and 71.1% sensitivity, respectively, and were both 100% specific. The lower detection limit for the lytic tests was 4.2%-5.0% of cells; the GCT sensitivity for PNH erythrocytes was 2%-4%; the FCM sensitivity was <1.0% (Figure 1). They concluded that the FCM is the most sensitive of the 4 tests, that sephacryl-GCT...
is a good PNH screen, and that the HT and SLT are still useful, if properly standardized.

Another FCM area of PNH studies involves the use of inactivated fluorescently labeled bacterial toxins, which bind to the GPI-anchors. Positivity for these toxins on normal cells, or negativity for these toxins on PNH clonal cells, provides more information than mAb-binding to GPI-anchor proteins because negativity for at least 2 proteins, such as CD55 and CD59, is necessary to diagnose PNH. MacKenzie and colleagues studied binding by proaerolysin and aerolysin, both products of Aeromonas hydrophila, to RBC GPI-anchor proteins and the interference of glycoporphin with aerolysin binding. Difficulties with FLAER-related tests for the PNH RBC clone have been noted; it has been speculated that this problem relates to glycoporphin binding. Brodsky and colleagues developed the FLAER test for PNH. They showed that FLAER was more sensitive than conventional flow cytometry (FCM) for the detection of PNH in the WBC clone. Wang and colleagues derived a similar toxin from a different strain of Aeromonas hydrophila J-1 and called it “Toxin HEC (hemolytic, enterotoxigenicity, cytotoxicity).” They determined that PNH RBCs were much more resistant to lysis by this toxin than were normal RBCs or RBCs from other hemolytic anemias. Shin and colleagues developed a Clostridium septicum fluoresceinated inactive mutant product for FCM and identified its utility for PNH diagnosis. Sutherland and colleagues identified PNH WBC clones by simultaneous use of FLAER with CD33, CD45, and CD14 for neutrophils and monocytes, which detected 11.8% PNH clones in 536 samples tested, whereas CD59 alone for RBCs resulted in only 6.2% positivity for PNH. Moreover, the assay involving FLAER and mAbs always found a higher percentage of PNH cells than the CD59 RBC assay, suggesting a greater sensitivity for the FLAER test. Peghini and Fehr found excellent correlation between FCM and FLAER and believe the FLAER test has a sensitivity as low as 0.5%. Sutherland and colleagues reconfirmed this sensitivity range (0.5%-1.0%) for FLAER with mAbs.

Because PNH cytogenetic abnormalities are more common in GPI (+) than GPI (-) cells, Beier and colleagues developed a combined molecular-aerolysin/flow cytometric technique by which aerolysin was used to identify GPI (+) and GPI (-) WBCs. The authors found telomere length by fluorescent in-situ hybridization (FISH) for granulocytes was decreased from that of healthy donors (7.73 telomere fluorescence units (TFU) ± 0.23) in both the GPI (+) (6.88 ± 0.38 TFU) and GPI (-) (6.51 ± 0.33 TFU) cells of PNH patients.

Discussion

The relevant history regarding PNH clinical testing covers approximately three quarters of a century. The HT from the late 1930s was followed by the CLS and SLT after about 1 generation. The predominance of the complement-lytic RBC tests ended about a quarter of a century later, with the advent of FCM for mAbs to GPI-anchor proteins. In the following decade FLAER testing began to supplement PNH mAb-based FCM. The FLAER negativity reflects the global loss of GPI-anchors and is a more significant finding than the decrease or absence of a solitary mAb marker. Currently, FCM with mAbs for GPI-anchor proteins and FLAER assays dominate this diagnostic area.

Since 2003 other bacterial toxins have been discovered that are similar to aerolysin; the attraction of these bacterial products is that they bind directly to the GPI-anchor, as opposed to the GPI-anchor protein, suggesting a superiority to mAbs. The FLAER tests are limited due to RBC-binding...
interference; thus, FLAER is more useful for identifying the WBC clone, which is not affected by hemolysis or transfusion. However, FLAER negativity alone should not lead to a PNH diagnosis because FLAER was not classified as an analyte specific reagent (2006).39 These FLAER-related PNH FCM assays, as well as the continued application of FCM mAbs, reflect the predominant, recent growth in PNH laboratory diagnostic technology. Molecular-based (DNA, RNA, etc) PNH tests have not increased as much as might have been hoped. Furthermore, the complement-lytic methods, HT and SLT, have largely been replaced.

The study by Gupta and colleagues28 compares tests across both the complement RBC-lytic and FCM eras. If the estimate for the low detection level28,35,36 of about 0.75% of total cells for FCM/FLAER/mAb methods is used, a strong negative correlation between the lower level for PNH cell detection and percent PNH clone identification in samples can be identified (Figure 1). Thus, the PNH detection limit was decreased from <5.0% of total cells for lytic methods, to about 3.0% for the GCT, to <1.0% of total cells for FCM related methods, indicative of improved sensitivity. This graph implies the finding of 11.8% PNH WBC clones in samples studied by a FLAER mAb combination vs 6.2% PNH RBC clones by CD 59 alone (Figure 2)35 indicates the FLAER/mAb combination is more sensitive and would have a lower detection limit for WBC PNH cells than the CD 59 alone would have for RBC PNH cells.36 Because the PNH RBC clone is affected by both transfusion and hemolysis, there will generally be a greater percentage of PNH WBCs than PNH RBCs, thus the PNH WBC clone will generally be more readily detected than the PNH RBC clone. Thus, the comparison by Sutherland35 of FLAER for the WBC clone vs CD 59 for the RBC clone (Figure 2) might have been improved by comparing FLAER to CD 55 for WBCs.

Hevessy and colleagues conceived the “MFI rate,” which is derived by comparing the MFI of PNH cells to normal cells; they separated the cells into 3 groups, analogous to PNH I, II, and III RBCs, by MFI.26 The use of a mathematically derived parameter here, although appearing to increase sensitivity, may have reduced specificity and can introduce error due to the derivation itself.

In summary, the laboratory diagnostic methods for PNH have become increasingly sensitive and specific since 2003, so that the lower limit of PNH cell detection is below 1% of total cells, and possibly approaches 0.01% for dual-color analysis of RBCs.40 Occasionally, a clone can approach 100% of cells with increased associated symptoms, such as hemolysis and thrombosis. Unfortunately, molecular methods for PNH are not yet widely employed clinically; FCM employing mAbs to GPI-anchor proteins and FLAER methods are dominant and have replaced complement-mediated RBC-lytic methods. A “neo-antigen” to positively identify PNH cells by FCM, as opposed to the FCM identification of cells negative for CD 55, CD 59, and FLAER, as done presently, has not been identified.3 Paroxysmal nocturnal hemoglobinuria detection relevance has been influenced by recent estimates of a 15-year prevalence at 1.59/100,000 and incidence at 1/100,000 cases to 5/1,000,000 births.31,42 A thorough review of state-of-the-art flow cytometric PNH diagnostic technique has recently appeared.43


