Differential Usefulness of Various Markers in the Flow Cytometric Detection of Paroxysmal Nocturnal Hemoglobinuria in Blood and Bone Marrow

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Key Words: Paroxysmal nocturnal hemoglobinuria; Flow cytometry; CD59; Blood; Bone marrow

Abstract

In this study, we examined the usefulness of various markers on blood cell populations in the diagnosis of paroxysmal nocturnal hemoglobinuria (PNH). We also evaluated bone marrow specimens, which generally are considered less suitable than blood owing to variable expression of glycosyl phosphatidylinositol (GPI)-linked antigens during hematopoietic cell differentiation. All 15 patients in our cohort had subpopulations of CD16/CD55-deficient granulocytes and CD14/CD55-deficient monocytes (“PNH clones”). The PNH clone size of granulocytes and monocytes was greater than that of erythrocytes or lymphocytes in the majority of the cases. It is interesting that CD59 showed limited usefulness for detecting PNH+ monocytes. Normal monocytes exhibited significantly dimmer CD59 expression than normal granulocytes. PNH-deficient monocytes expressed only marginally lower CD59, making it a less robust marker for highlighting PNH+ monocytes compared with CD14 or CD55. Finally, our study demonstrated the definitive usefulness of a limited combination of markers (CD16/CD55/CD45/CD14) in detecting GPI-deficient monocytes and granulocytes in bone marrow specimens submitted for flow cytometric evaluation of cytopenias.

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare, acquired clonal disorder in which patients have complement-mediated hemolytic anemia. A propensity toward venous thromboses and cytopenias due to bone marrow failure complete the triad of clinical symptoms. PNH is the result of a somatic mutation arising in the pig-a gene of the hematopoietic stem cell. This causes a change in the biosynthetic pathway of glycosyl phosphatidylinositol (GPI) moieties, which anchor various proteins on the cell surface following posttranslational modification. Thus, GPI-linked proteins that normally are present on the blood cell surface are abrogated in patients with PNH. The lack of CD55 (decay accelerating factor) and CD59 (membrane inhibitor of reactive lysis) in RBC membranes is responsible for unchecked complement activation and subsequent chronic hemolytic anemia with episodic exacerbation.

Traditionally, the acid hemolysis (Ham test) and sucrose lysis tests were used for identifying PNH erythrocytes. However, they are not reliable for assessing small PNH clones, and the results are confounded by recent transfusions. The complement lysis sensitivity test is more accurate for characterizing the RBC populations but is not used routinely owing to its technical complexity. Therefore, flow cytometry (FC) has become the “gold standard” for detecting and quantifying PNH clones. Most flow cytometric methods use fluorescent-labeled monoclonal antibodies to detect various GPI-anchored surface antigens. Other reported techniques use a mutant variant of the bacterial toxin, aerolysin, that binds to GPI molecules. Despite the wealth of diagnostic information available, there are no standardized criteria for use of FC in the diagnosis of PNH.

We evaluated the performance of various immunophenotypic markers in different blood cell populations for the
diagnosis of PNH. Although peripheral blood is preferred for detecting a PNH clone owing to uniform expression levels of GPI-linked antigens on normal mature blood cells, bone marrow is submitted more frequently for flow cytometric analysis of unexplained cytopenias. Therefore, we also examined the usefulness of bone marrow specimens in evaluation for PNH.

Materials and Methods

The University of Texas Southwestern Medical Center Flow Cytometry Laboratory (Dallas) database was searched for cases with positive PNH clones between April 1997 and August 2005. We identified 15 cases. Clinical data were obtained through review of medical records. The study was approved by the University of Texas Southwestern Review Board.

All 15 patients had a blood sample immunophenotyped with a PNH panel, including antibodies against CD3, CD14, CD16, CD19, CD22, CD33, CD45, CD55, and CD59. Blood specimens from 15 concurrently drawn healthy control subjects also were analyzed with the panel. Of 15 patients, 8 had a bone marrow biopsy analyzed with a bone marrow screen panel at the same time. The bone marrow screen panel consisted of antibodies to CD3, CD4, CD7, CD8, CD10, CD11b, CD14, CD16, CD19, CD20, CD22, CD36, CD38, CD45, CD56, CD64, κ, and λ. To assess the usefulness of bone marrow for diagnosis of PNH, a screening panel of CD16/CD55/CD45/CD14 was performed prospectively on marrow from 2 patients with PNH and 10 normal control marrow samples.

For preparation of single-cell suspensions for 4-color FC, peripheral blood or bone marrow specimens were incubated for 10 minutes with a standard ammonium chloride lysing solution (1 part sample/5 parts lysing solution). The samples then were washed twice with phosphate-albumin buffer (PAB), 0.0455% sodium azide, and 0.1% bovine serum albumin solution and resuspended in 5% fetal calf serum in RPMI 1640 culture medium. An aliquot of 5 × 10⁵ cells was stained with a combination of 4 antibodies conjugated with fluorescein isothiocyanate, phycoerythrin, peridinin chlorophyll protein, or allophycocyanin at 2°C to 8°C in the dark for 20 minutes. The amount of antibody added was based on the manufacturer’s recommendations. For RBC analysis, whole blood samples were diluted 1:100 in PAB and stained with monoclonal antibodies for 25 minutes at room temperature (20°C-25°C) in the dark. The stained cells were washed with PAB and resuspended in 1% paraformaldehyde in phosphate-buffered saline. At least 30,000 events were acquired routinely using a FACSCalibur flow cytometer with CellQuest software (Becton Dickinson, San Jose, CA). Data analysis was performed using Paint-A-Gate software (Becton Dickinson).

The following antibodies were obtained from Becton Dickinson, unless otherwise specified: CD3 (SK7), CD4 (SK3), CD7 (4H9), CD8 (SK1), CD10 (W8E7), CD11b (D12), CD14 (MP9), CD16 (NKP15), CD19 (SJ25C1), CD20 (L27), CD22 (SHCL-1), CD24 (ML5), CD36 (FA6.152; Beckman Coulter, Miami, FL), CD38 (HB7), CD45 (2D1), CD55 (IA10), CD56 (MY31), CD64 (10.1; Caltag, Burlingame, CA), CD59 (p282/H19), polyclonal κ and polyclonal λ (goat IgG; Beckman Coulter). In general, distinct cell populations (granulocytes, monocytes, lymphocytes, and erythrocytes) were identified by using CD45/forward and orthogonal light scatter characteristics. For accurate enumeration of GPI-deficient monocyte populations, monocytes were identified by analysis of light scatter properties in combination with CD45 and CD33. In addition, B-lymphocytes were discriminated from other small lymphocytes based on the expression pattern of CD3 and CD19.

The size of a PNH clone in each lineage was determined by a shift of a subpopulation from normal cell type in the expression of 2 GPI-linked antigens. An antigen was considered decreased if the cluster of interest fell below a 98% normal control threshold. The partial or complete absence of 1 or more GPI-linked antigens in 2 or more different lineages is indicative of PNH. A PNH clone size of more than 3% for RBCs and more than 10% for granulocytes was considered suggestive of classic PNH, whereas smaller clone sizes were “suspicious” for hypoplastic PNH.

Statistical analyses of data were carried out in Origin (OriginLab, Northampton, MA) and Excel (Microsoft, Redmond, WA). A P value of less than .05 was considered statistically significant.

Results

Clinical and Laboratory Characteristics

The cohort consisted of 8 men and 7 women with a mean age of 33.8 years (range, 14-62 years). All patients had cytopenias of variable severity. Mild to moderate anemia was noted in 13 cases (87%), leukopenia in 11 (73%), and thrombocytopenia in all 15 (100%). Thirteen patients had a clinical diagnosis of PNH, 1 had hepatitis C, and 1 had a history of aplastic anemia. The patients’ pertinent laboratory data are summarized in Table 1.

Flow Cytometric Analysis of PNH+ Blood Cells

Two GPI-linked antigens, CD55 and CD59, were used to assess the PNH+ erythrocytes. Two GPI-linked antigens, CD55 and CD59, were used to assess the PNH+ erythrocytes in the 15 healthy control subjects, a minor population (0.40% ± 0.20%) of erythrocytes deficient in CD55 and CD59 was detected. The size for CD55/CD59-deficient erythrocytes was even smaller (0.05% ± 0.01%) among 11 control samples that were analyzed within the last 6 years. Both antigens were deficient in similar levels in erythrocytes in 11 of 13 assessed cases in Table 2. In
2 patients, RBC populations lacked surface expression of only 1 of these antigens (Table 2). The size of the detected PNH clones ranged from 0.07% to 95% (mean ± SE, 36% ± 10%) and was 4.2% or more in 11 of 13 patients.

A combination of CD14, CD16, CD24, CD55, and CD59 was used to establish the proportion of GPI-deficient granulocytes. CD16 and CD55 were equally reliable in providing an accurate representation of the PNH clone; their combination identified a distinct population of PNH+ granulocytes (mean ± SE, 43% ± 9.5%) in all cases (Image 1). The size of the CD24-deficient granulocyte clone (43% ± 10%) matched that of the combination of CD16 and CD55. In contrast, CD59 and CD14 highlighted a smaller PNH clone (31% ± 9.2% and 32% ± 10%, respectively) compared with the other markers. The size for CD16/CD55-deficient granulocytes in the control group was 0.12% ± 0.07%.

**Table 1**

<table>
<thead>
<tr>
<th>Laboratory Value</th>
<th>Mean ± SE</th>
<th>Reference Range</th>
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<tbody>
<tr>
<td>Hemoglobin, g/dL (g/L)</td>
<td>10.0 ± 0.59 (100 ± 5.9)</td>
<td>12.1-16.1 (121-161)</td>
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<td>Mean corpuscular volume, µm³ (fL)</td>
<td>95 ± 2.7 (95 ± 2.7)</td>
<td>74-102 (74-102)</td>
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<td>Reticulocytes, x 10⁹/µL (x 10⁹/L)</td>
<td>130 ± 44 (130 ± 44)</td>
<td>320-1,470 (320-1,470)</td>
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<td>WBCs, /µL (x 10⁹/L)</td>
<td>3,900 ± 710 (3.9 ± 0.71)</td>
<td>3,900-10,700 (3.9-10.7)</td>
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<tr>
<td>Platelets, x 10³/µL (x 10⁹/L)</td>
<td>68 ± 17 (68 ± 17)</td>
<td>174-404 (174-404)</td>
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<tr>
<td>Lactate dehydrogenase, U/L</td>
<td>360 ± 49</td>
<td>100-190</td>
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<tr>
<td>Haptoglobin, mg/dL (g/L)</td>
<td>18 ± 9.3 (0.18 ± 0.093)</td>
<td>30-200 (0.3-2.0)</td>
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<tr>
<td>Total bilirubin, mg/dL (µmol/L)</td>
<td>1.2 ± 0.25 (21 ± 4)</td>
<td>0.2-1.3 (3-22)</td>
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</table>

**Image 1** Detection of paroxysmal nocturnal hemoglobinuria (PNH)+ blood cells by flow cytometric analysis. Normal blood cells are painted in red (erythrocytes), blue (monocytes), and green (granulocytes). Distinct subpopulations of CD55–/CD59– RBCs (black), CD14–/CD55(dim) monocytes (violet), and CD16(dim)/CD55(dim) granulocytes (yellow) are identified in a blood sample from a patient with clinical diagnosis of PNH.
Of the GPI-linked monocyte antigens (Figure 1), CD14 and CD55 generally were lacking on similar numbers of PNH+ monocytes: 44% ± 9.6% and 47% ± 10%, respectively. The population size of CD14/CD55-deficient monocytes in the control group was 0.13% ± 0.08%. By using these 2 markers, a distinct population of PNH+ monocytes was identified in all 15 patients with PNH (Image 1). The clone size was similar to that in granulocytes and greater than that in erythrocytes in the majority of cases (Table 2). It is interesting that CD59-deficient monocyte populations were considerably smaller (17% ± 9%), but the difference in mean fluorescence intensity of CD59 in normal and deficient monocytes was still statistically significant (105 ± 14 vs 65 ± 11, \( P = .039 \)). The low sensitivity of CD59 for PNH+ monocytes was largely due to the low surface CD59 density. Normal monocytes showed significantly dimmer CD59 expression than normal granulocytes (105 ± 14 vs 173 ± 25, \( P = .025 \)). The change of CD59 mean fluorescence intensity in PNH+ monocytes was significantly smaller than that for PNH+ granulocytes (43 ± 10 vs 146 ± 23; \( P = .0005 \))

Table 2

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Clinical Diagnosis</th>
<th>Erythrocytes</th>
<th>Granulocytes</th>
<th>Monocytes</th>
<th>B Lymphocytes</th>
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<tr>
<td>1</td>
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<td>58</td>
<td>49</td>
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<td>2</td>
<td>PNH</td>
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<td>35</td>
<td>61</td>
<td>24</td>
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<td>PNH</td>
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<td>95</td>
<td>87</td>
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<tr>
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<td>PNH</td>
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<td>68</td>
<td>63</td>
<td>3.5</td>
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<tr>
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<td>PNH</td>
<td>U†</td>
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<td>48</td>
<td>50</td>
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<tr>
<td>12</td>
<td>Aplastic anemia</td>
<td>0.1</td>
<td>1.7</td>
<td>1.6</td>
<td>5.7</td>
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<tr>
<td>13</td>
<td>Hepatitis C</td>
<td>0.07</td>
<td>3.4</td>
<td>2.5</td>
<td>2.8</td>
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<tr>
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<td>PNH</td>
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<td>7.8</td>
<td>1.1</td>
<td>17</td>
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<tr>
<td>15</td>
<td>PNH</td>
<td>NA</td>
<td>7.9</td>
<td>6.5</td>
<td>8.3</td>
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</tbody>
</table>

NA, not available; PNH, paroxysmal nocturnal hemoglobinuria.

* 50% CD55–/CD59+ RBCs.
† 100% CD55+/CD59– RBCs.

In the analysis of PNH+ lymphocytes, we focused on the characterization of B cells using antibodies to CD19, CD24, and CD55. The clone size of PNH+B cells was generally

![Figure 1](image1.png) Clone size of glycosyl phosphatidylinositol (GPI)-deficient blood monocytes and granulocytes by various markers.

![Figure 2](image2.png) Comparison of mean fluorescence intensity of CD59 in normal and paroxysmal nocturnal hemoglobinuria (PNH)+ monocytes and granulocytes. The dotted lines match normal cells with PNH+ cells in the same sample.
smaller than those in other lineages (Table 2). The proportion of GPI-deficient B lymphocytes ranged from 0.4% to 13% for CD55 (mean ± SE, 2.4% ± 1.1%) and from 0% to 68% for CD24 (mean ± SE, 17% ± 5.1%). The sizes for CD55- and CD24-deficient B lymphocytes in the control group were 0.74% ± 0.17% and 2.77% ± 0.64%, respectively. Notably, there is a wide range of CD24 density on normal B lymphocytes. Thus, CD24 alone is not sufficient to define a small population of PNH+ B cells.

Flow Cytometric Analysis of PNH+ Marrow Granulocytes and Monocytes

Eight patients had bone marrow samples submitted for FC for evaluation of blood cytopenias before having PNH populations detected in their peripheral blood. Immunophenotypic analysis of these marrow samples identified abnormal expression patterns of CD14 on monocytes and CD16 on granulocytes in 3 (38%) and 5 (63%) cases, respectively. The average size of the GPI-deficient clone was 75% (range, 48%-95%) in monocytes and 70% (range, 21%-99%) in granulocytes.

To reliably evaluate PNH clones in bone marrow samples, we combined CD14, CD16, and CD45 with CD55 for detecting PNH+ monocytes and granulocytes. The coexpression patterns of CD55 and CD14 or CD16 during hematopoietic cell differentiation were characterized in 10 normal bone marrow samples. The majority of marrow monocytes expressed bright CD55, whereas marrow granulocytes showed a gradual increase in CD55 during maturation. However, no distinct populations of CD14/CD55-deficient monocytes or CD16/CD55-deficient granulocytes were observed in any of the normal marrow samples. In contrast, discrete populations of CD14/CD55-deficient monocytes and CD16/CD55-deficient granulocytes were identified in both available marrow samples from 2 patients with an established diagnosis of PNH. 

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**Image 2**: Abnormal expression patterns of CD14 and CD16 in paroxysmal nocturnal hemoglobinuria (PNH)+ marrow monocytes and granulocytes. **A**, Expression patterns of CD14, CD16, and CD55 in normal marrow monocytes (blue) and granulocytes (green). **B**, Lack of CD14 on monocytes and CD16 on granulocytes in a representative PNH marrow.
Discussion

FC is considered the gold standard for detecting GPI-deficient blood cells. Traditionally, CD55 and CD59 are commonly used markers in the analysis of PNH. Availability of monoclonal antibodies to other GPI-linked antigens greatly enhances the power of detection. However, there has been no consensus on antibody selection or immunophenotypic criteria for a diagnosis of PNH. Some authors advocate detection of at least 2 missing GPI-linked antigens on 2 different cell lines for determining the presence of a PNH clone.\textsuperscript{21,22} Clinically, this entity does not always manifest with the hallmark feature of transient intravascular hemolysis, but rather with the clinical picture dominated by cytopenias. The different clinical manifestations led to the distinction between “classic” or hemolytic PNH and “hypoplastic” or aplastic anemia–associated PNH.\textsuperscript{7,23} In addition, this classification also is based on the relative size of a PNH clone. In this respect, the cutoff is not very clear, although values of 3% or less GPI-deficient RBCs for hypoplastic PNH and 7% or more for classic PNH have been proposed.\textsuperscript{23}

We undertook the present study to evaluate the flow cytometric efficacy of various markers in the detection of a PNH clone. Among the markers in our panels, CD14, CD16, CD24, CD55, and CD59 all were effective in the detection of PNH+ granulocytes, although the combination of CD16 and CD55 was most sensitive in estimating a GPI-deficient clone. Conversely, CD59 showed limited usefulness for PNH+ monocytes, which exhibited only slightly diminished levels of CD59 in 13 of 15 cases. Similar observations were described previously.\textsuperscript{16,20} However, our study further demonstrated that the low sensitivity of CD59 for PNH+ monocytes

![Image 3](Image3.png)

**Image 3** Detection of paroxysmal nocturnal hemoglobinuria (PNH)+ marrow monocytes and granulocytes by a screening panel of CD16/CD55/CD45/CD14. **A**, Coexpression patterns of CD14/CD55 in monocytes (blue) and CD16/CD55 in granulocytes (green) during normal hematopoietic cell differentiation. **B**, Distinct subpopulations of PNH+ monocytes (violet) and PNH+ granulocytes (yellow) in a bone marrow sample from a patient with a clinical diagnosis of PNH.
was attributed to a generally low density of surface CD59 in monocytes. CD59 expression was significantly lower in normal monocytes compared with normal granulocytes. Therefore, PNH+ monocytes were best characterized by the combination of CD14 and CD55. All 15 patients from our cohort had subpopulations of CD16/CD55-deficient granulocytes and CD14/CD55-deficient monocytes.

In the present study, the proportion of PNH+ granulocytes and/or monocytes was greater than that of the PNH+ erythrocytes or lymphocytes in the majority of our patients. A smaller size of the erythrocyte PNH clone can be explained by the shortened life span of PNH RBCs and/or recent blood transfusion, whereas a smaller size of the lymphocyte PNH clone is likely due to a long life span of normal lymphocytes generated before the onset of PNH.17 PNH clone size varied from 11% to 99% (mean, 64%) in 13 of our patients with a clinically established diagnosis of PNH, and was less than 5% in the remaining 2 patients with aplastic anemia or hepatitis C. Based on these results, a clone size greater than 10% by FC is suggestive of PNH.

PNH may masquerade clinically as myelodysplasia, leukemia, or lymphoma. Thus, the first sample from a patient submitted for flow cytometric analysis could be a bone marrow aspirate. Because CD14 and CD16 usually are included in a panel for flow cytometric analysis of bone marrow, we examined the efficacy of these antibodies in the detection of CD14-deficient monocytes and CD16-deficient granulocytes in the marrow samples of 8 patients with PNH. Abnormal patterns of CD14 expression on monocytes and/or CD16 expression on granulocytes were identified in 5 (63%) of 8 marrow samples. Notably, the abnormal expression patterns of these antigens were reported to be 100% sensitive for PNH monocytes and granulocytes in a prior study of 15 cases.19 This discrepancy might be related to different PNH clone sizes in marrow. Although the clone sizes were not reported in the previous study, a clone size less than 21% was undetectable using CD14 and CD16 in our study. On the other hand, decreased expression of CD14 and CD16 is not specific for PNH+ monocytes and granulocytes and can be seen in myelodysplastic syndromes and other conditions.24,25 Furthermore, a shift of monocytes or granulocytes toward immaturity is accompanied by reduced CD14 or CD16 expression. Apoptotic granulocytes may partially lose CD16,26 and neutrophils from people with a polymorphic variant of CD16 are not recognized by commercially available monoclonal anti-CD16.7 These observations support the importance of combining information on expression of more than 1 GPI-linked antigen in different lineages for establishing a diagnosis of PNH.

We prospectively evaluated the usefulness of a screen tube of CD16/CD55/CD45/CD14 for the detection of PNH+ marrow monocytes and granulocytes. Although immature monocytes and granulocytes usually exhibit a slightly lower level of surface CD55 than mature ones, there is no subpopulation of dual CD14/CD55-deficient monocytes or CD16/CD55-deficient granulocytes in normal marrow. By using a CD16/CD55/CD45/CD14 tube, distinct subpopulations of PNH+ marrow monocytes and granulocytes were identified easily in the 2 patients with PNH, supporting the diagnostic usefulness of this panel for flow cytometric analysis of bone marrow samples in patients with unexplained cytopenia. This simple screening panel might constitute a rapid and cost-effective indicator for further confirmatory testing of PNH, a disease that can be diagnostically challenging owing to its low incidence and variable clinical manifestations.

References


