The Use of Monoclonal Antibodies and Flow Cytometry in the Diagnosis of Paroxysmal Nocturnal Hemoglobinuria

By Sharon E. Hall and Wendell F. Rosse

We have characterized the erythrocytes, granulocytes, and platelets of 54 patients with paroxysmal nocturnal hemoglobinuria (PNH) with antibodies to glycosylphosphatidylinositol-anchored proteins (anti-CD55, anti-CD59, and anti-CD16) and flow cytometry to establish the usefulness of this technique in the diagnosis of this disorder. All patients demonstrated either completely (PNH III) or partially (PNH II) deficient red cells and granulocytes. Anti-CD59 best demonstrated PNH II red cells, which were present in 50% of the patients. The proportion of abnormal granulocytes was usually greater than the proportion of abnormal red cells; 37% of the patients had >80% abnormal granulocytes. Anti-CD55 did not delineate the erythrocyte populations as well as did anti-CD59. Either anti-CD55 or anti-CD59 could be used equally well to analyze granulocytes; anti-CD16 did not demonstrate cells of partial deficiency. Platelets could not be used for detailed analysis as the normal and abnormal populations were not well distinguished. Flow cytometry of erythrocytes using anti-CD59 or of granulocytes using either anti-CD55 or anti-CD59 provides the most accurate technique for the diagnosis of paroxysmal nocturnal hemoglobinuria; it is clearly more specific, more quantitative, and more sensitive than the tests for PNH that depend upon hemolysis by complement (the acidified serum lysis [Ham] test, the sucrose lysis test, and the complement lysis sensitivity [CLS] test).

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**Materials**

The MoAb IH4, specific for CD55, was produced in our laboratory. An MoAb to CD59, 10G10, was the gift of Dr Marilyn Telen (Duke University, Durham, NC). An MoAb to CD16 (3G8) was the gift of Dr Mark Currie. Mouse anti-human glycoporphin A antibody labeled with R-phycocerythrin (R-PE) was purchased from Pharmingen (San Diego, CA). An MoAb, CD13 directly labeled with R-PE was obtained from Souther Biotechnology Associates, Inc (Birmingham, AL). Nonreactive MoAb P3 with random specificity was used as a negative control. Fluorescein-conjugated goat F(ab')2 anti-mouse IgG (FITC) was purchased from Biorad Laboratories (Camarillo, CA). Normal goat serum and Dulbecco's phosphate-buffered saline (PBS) were purchased from Gibco BRL (Grand Island, NY). Human serum was obtained from normal healthy group AB volunteers. Ortho-mune lysing reagent was purchased from Ortho Diagnostic Systems Inc (Raritan, NJ). Dextran T500 was obtained from Pharmacia Biotech Inc (Piscataway, NJ). Lymphocyte Separation Medium (LSM) was purchased from Orgonon Teknika Corporation (Durham, NC). The buffer used in the flow cytometry assays were veronal-buffered saline (GVB-EDTA), composed of 0.005 mol/L sodium barbital, 0.15 mol/L NaCl, 0.1% gelatin, 0.015 mol/L EDTA, pH 7.5.

**Preparation of Cells for Flow Cytometry**

Venous peripheral blood was drawn from patients with PNH or suspected of having the diagnosis of PNH was tested. The patients ranged in age from 11 to 65 years (mean 34 years). Erythrocytes and unseparated granulocytes of 54 patients, purified granulocytes of 39 patients, and platelets of 18 patients were tested by flow cytometry. Samples of blood from healthy adult volunteers were analyzed concurrently to determine normal values.
The platelet-rich plasma was then centrifuged at 1500 rpm for 5 minutes, and the platelet pellet was washed twice and resuspended to $1 \times 10^9$/mL in GVB-EDTA.

**Immunophenotype Analysis**

Single color flow analysis was performed by using a two-step method. One hundred microliters of the various cell suspensions were incubated with 100 μL of each MoAb and P3 for 30 minutes at room temperature. The cells were then washed twice with GVB-EDTA and resuspended to 100 μL volume. A 10-μL mixture of GAM-FITC, normal goat serum, and group AB normal human serum (ratio 1:1:2.5) was added and incubated for another 30 minutes at room temperature, followed by two washes in GVB-EDTA. The pelleted cells were resuspended in 1 mL of buffer and analyzed on an Ortho Cytoron Absolute (Ortho Diagnostic Systems Inc). Cell types were determined by their forward and right scatter.

Two-color analysis was performed as above except that MoAbs to specific cell markers and directly labeled with R-PE were used at the recommended manufacturer’s dilutions as a third step. Color compensation for crossover of fluorescence signals between the two detectors was adjusted for optimal analysis.

**Complement Lysis Sensitivity Test (CLS)**

A modified version of the original CLS test of Rosse and Dacie was used to compare erythrocyte populations to flow cytometry. The reaction volume was reduced to 375 μL with the proportions of erythrocytes, complement, and buffer staying the same. PNH cells give complex curves that are analyzed to determine the three different PNH cell populations.

**RESULTS**

**Erythrocytes**

**Normal erythrocytes.** The erythrocytes from 44 normal donors were examined with anti-CD55 and those of 49 normal donors with anti-CD59 (Table 1). The mean fluorescence was greater with anti-CD59. Cells not reacting with the antibody used (unstained cells) were present in small numbers in each case.

Erythrocytes from two normal donors were maintained at 4°C for up to 25 days to determine the stability of the detection of CD59. Six replicate samples were analyzed at each point for percentage of cells bearing CD59 and for the mean fluorescence obtained under standardized conditions. The percentage of cells bearing CD59 remained stable in time (range 98.3% to 99.8% and 98.4% to 99.7%, respectively) and did the standard deviation of the determination (range 0.03% to 0.94%). The mean fluorescence decreased with time (Fig 1).

**PNH erythrocytes.** The washed erythrocytes from 54 patients with the diagnosis of or suspected of having the diagnosis of PNH were examined by flow cytometry with anti-CD59 and anti-CD55. Using anti-CD59, three types of cells could be distinguished (Fig 2): cells with nearly normal expression of GPI-anchored protein (PNH I cells), cells with no detectable expression of GPI-anchored proteins (PNH III cells), and cells with intermediate expression of GPI-anchored proteins (PNH II cells). Cells of intermediate expression occurred either as a discrete population (Fig 2c), as “transition” between PNH III and PNH I cells (Fig 2d), or as an indistinct major population (Fig 2f). The mean fluorescence using anti-CD59 of these populations is shown in Fig 3. An intermediate population was more readily seen when anti-CD59 was used, in part because the mean fluorescence of the PNH I cells is greater than with anti-CD55.

The proportion of cells in each erythrocyte population was analyzed and the results for anti-CD59 are shown in Table 2. The pattern of populations of PNH erythrocytes for 54 patients is shown in Table 3.

In 38 patients, the results of flow cytometry analysis were compared to the results of the CLS test. The results were concordant in 25 of 38 patients (65.8%) in that both identified the same populations, although the proportion of cells in the populations may not have been entirely similar. The CLS test did not detect a very small abnormal population of cells in one patient, whereas no patients tested had abnormal cells by CLS and none by flow cytometry. In the other 12 patients (31.2%), the CLS test did not identify correctly the degree of abnormality (PNH I v PNH II v PNH III) or did not identify small populations (particularly small populations of PNH II cells).

To determine the reproducibility and stability of the determinations of CD59 on PNH erythrocytes, the blood of three patients was maintained at 4°C for up to 25 days and was tested at intervals, analyzing six replicate samples at each

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**Table 1. Analysis of Normal Erythrocytes by Flow Cytometry and Anti-CD55 and Anti-CD59**

<table>
<thead>
<tr>
<th></th>
<th>Anti-CD55</th>
<th>Anti-CD59</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of samples</td>
<td>44</td>
<td>49</td>
</tr>
<tr>
<td>Mean fluorescence (channel)</td>
<td>$76.8 \pm 12.1$</td>
<td>$137.5 \pm 21.2$</td>
</tr>
<tr>
<td>Unstained cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of samples*</td>
<td>20 (45)</td>
<td>41 (83.7)</td>
</tr>
<tr>
<td>Mean percentage</td>
<td>$0.23 \pm 0.34$</td>
<td>$0.31 \pm 0.39$</td>
</tr>
<tr>
<td>Range (%)</td>
<td>0–1.4</td>
<td>0–1.6</td>
</tr>
</tbody>
</table>

* Values in parentheses are percentages.
Normal granulocytes. The granulocytes, separated or unseparated, from normal donors were examined with anti-CD55 and anti-CD59 (Table 4). In many donors, unstained cells were found; a larger proportion of these cells were found when unseparated granulocytes were examined, particularly when anti-CD59 was used in analysis.

PNH granulocytes. The separated and unseparated granulocytes of 35 patients with PNH or suspected of having PNH were analyzed using anti-CD55, anti-CD59, and anti-CD16 (Fig 5a through f). The cells could be divided into populations of PNH I, PNH II, and PNH III, using criteria.
DIAGNOSIS OF PNH BY FLOW CYTOMETRY

Table 2. Percentage of Abnormal Cells in Each of the Three PNH Populations in Erythrocytes and Isolated Granulocytes

<table>
<thead>
<tr>
<th>Percent</th>
<th>Erythrocytes (Anti-CD59)</th>
<th>Granulocytes (Anti-CD55)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PNH I</td>
<td>PNH II</td>
</tr>
<tr>
<td>0–20</td>
<td>9 (16.7)</td>
<td>15 (26.8)</td>
</tr>
<tr>
<td>20–40</td>
<td>15 (27.8)</td>
<td>3 (5.4)</td>
</tr>
<tr>
<td>40–60</td>
<td>8 (14.8)</td>
<td>1 (1.8)</td>
</tr>
<tr>
<td>60–80</td>
<td>15 (27.8)</td>
<td>—</td>
</tr>
<tr>
<td>80–100</td>
<td>7 (13.0)</td>
<td>2 (3.6)</td>
</tr>
<tr>
<td>Total</td>
<td>54 (100)</td>
<td>21 (37.5)</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages.

by van der Schoot and associates in 1990 in the analysis of granulocytes with a panel of antibodies including anti-CD16. These studies were confirmed by Schubert et al; in both studies, the totally deficient and normal populations of cells were found in all blood elements but partially deficient cells were not identified. Red cells of intermediate abnormality were identified in flow cytometry by Rosse et al and Shichishima et al, and, in both studies, were compared with the results obtained by the CLS test. More recently, Kwong et al have indicated other antibodies that may be used in detecting GPI-deficient cells in PNH. In none of these studies were the antibodies used compared in a large number of patients to establish the optimal conditions and techniques that should be used in the diagnosis of PNH and characterization of the cells in this disorder.

In the present study, we have analyzed the cells of 54 patients with PNH to establish the optimum conditions for using flow cytometry in the diagnosis of the disorder. We have found that the analysis of erythrocytes is best carried out using anti-CD59. The mean fluorescence obtained with the GPI-positive cells is sufficiently great as to be readily distinguished from the GPI-negative cells; this was not the case with anti-CD55 (anti-DAF). The mean fluorescence of the GPI-positive PNH I cells in PNH was significantly less similar to those used for analyzing red cells. The separation of the populations and the ability to analyze accurately their percentage was greater with separated than unseparated cells. The mean fluorescence of the cells in each population is shown for separated cells in Fig 6. The proportion of cells in each population for 35 patients is shown in Table 2. The pattern of populations of PNH granulocytes for 35 patients is shown in Table 3.

The relationship between the proportion of cells in each population to the proportion of cells in the corresponding red cell population is shown in Fig 7. In 23 of 35 cases (65.7%), the proportion of abnormal granulocytes was greater than the proportion of abnormal red cells, in 7 cases (20.0%) not significantly different, and in 5 cases (14.3%), it was less.

Anti-CD16 also distinguished PNH granulocytes but did not distinguish populations of intermediate content (PNH II cells).

Normal and PNH Platelets

Normal and PNH platelets were tested with anti-CD55 and anti-CD59. The amount of both proteins detected on the surface of normal cells was small with the result that the populations of cells in PNH patients were not well distinguished.

DISCUSSION

The use of MoAbs to GPI-linked proteins and flow cytometry in the definition of the defect in PNH was first proposed by van der Schoot and associates in 1990 in the analysis of granulocytes with a panel of antibodies including anti-CD16. These studies were confirmed by Schubert et al; in both studies, the totally deficient and normal populations of cells were found in all blood elements but partially deficient cells were not identified. Red cells of intermediate abnormality were identified in flow cytometry by Rosse et al and Shichishima et al, and, in both studies, were compared with the results obtained by the CLS test. More recently, Kwong et al have indicated other antibodies that may be used in detecting GPI-deficient cells in PNH. In none of these studies were the antibodies used compared in a large number of patients to establish the optimal conditions and techniques that should be used in the diagnosis of PNH and characterization of the cells in this disorder.

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Fig 4. Changes in the percent of abnormal (PNH II [open symbols] and/or PNH III [closed symbols]) cells with time in storage in plasma at 4°C. Each point is the mean of six replicate determinations; 1 SD is indicated by the bars associated with it.

Table 3. Patterns of Populations of Erythrocytes and Granulocytes in the Blood of Patients with PNH

<table>
<thead>
<tr>
<th>Populations*</th>
<th>Erythrocytes (Anti-CD59)</th>
<th>Granulocytes (Anti-CD55)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNH I + II</td>
<td>—</td>
<td>1 (2.9)</td>
</tr>
<tr>
<td>PNH I + II + III</td>
<td>15 (27.8)</td>
<td>2 (5.7)</td>
</tr>
<tr>
<td>PNH I + III</td>
<td>6 (11.1)</td>
<td>1 (2.9)</td>
</tr>
<tr>
<td>PNH I + III</td>
<td>27 (50)</td>
<td>10 (51.4)</td>
</tr>
<tr>
<td>PNH I</td>
<td>—</td>
<td>1 (2.9)</td>
</tr>
<tr>
<td>PNH II + III</td>
<td>2 (3.6)</td>
<td>2 (5.7)</td>
</tr>
<tr>
<td>PNH III</td>
<td>1 (2.9)</td>
<td></td>
</tr>
<tr>
<td>PNH I + II + III</td>
<td>4 (7.1)</td>
<td>9 (25.7)</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages.

* Parentheses indicate that the population is indistinct or transitional.
Table 4. Analysis of Isolated and Nonisolated Normal Granulocytes by Flow Cytometry and Anti-CD55 and Anti-CD59

<table>
<thead>
<tr>
<th>Separated Granulocytes</th>
<th>Unseparated Granulocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-CD55</td>
</tr>
<tr>
<td>No. of samples</td>
<td>15</td>
</tr>
<tr>
<td>Mean fluorescence (channel)</td>
<td>119.7 ± 7.6</td>
</tr>
<tr>
<td>Unstained cells</td>
<td></td>
</tr>
<tr>
<td>No. of samples*</td>
<td>6 (40)</td>
</tr>
<tr>
<td>Mean percentage</td>
<td>0.15 ± 0.25</td>
</tr>
<tr>
<td>Range (%)</td>
<td>0–0.9</td>
</tr>
</tbody>
</table>

*Values in parentheses are percentages.

than that of normal cells, a result also obtained by Shichishima et al; this suggests that the PNH I cells of PNH may not be entirely normal in their content of GPI-linked proteins.

A very small number of cells not reacting with the antibodies was found in the blood of a few normal control subjects. Analysis by two-color cytometry as well as repeated analyses from the same patient suggested that these did not represent a small population of PNH-like cells. Their presence did, however, set the lower limit of detection of a PNH population at about 1% of the cells.

Red cells of intermediate expression of GPI-dependent proteins were more readily identified using anti-CD59 than anti-CD55. These appeared to occur in three patterns: a distinct population of cells (17 patients), a continuum of cells between the PNH I and III cells (8 patients) and as a majority of the cells with deficient and nearly normal cells at each end of the spectrum (2 patients). All in all, 27 of 54 patients were found to have cells of intermediate expression. These findings correspond well with the findings using special tests of complement sensitivity and cell separation. However, when compared with the complement lysis sensitivity test alone, PNH II cells were more readily identified by flow cytometry.

These studies demonstrate the degree of variability of the determination of the fraction of PNH cells in each population and the amount of CD59 on the membrane (by determination of the mean fluorescence channel) by replicate sampling. In addition, they show that the amount of CD59 on normal cells and PNH I and II cells gradually decreases as the cell is stored; this protein may be digested by enzymes in the plasma or may dissociate from the membrane, as has been demonstrated for GPI-linked proteins. They also demonstrate that the fraction of abnormal cells consistently falls over time; the reason for this is not apparent. These two factors suggest that analysis is best performed with samples as fresh as possible.

The analysis of granulocytes for the deficiency of GPI-dependent proteins can also be used to diagnose PNH. All three antibodies used (anti-CD59, anti-CD55, and anti-CD16) readily demonstrated the deficiency of GPI-dependent proteins in some of the cells of all patients and the proportion detected by each was approximately the same.

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**Fig 5. Histograms of fluorescence of granulocytes using anti-CD59 and FITC-labeled anti-IgG.** (a) Normal granulocytes; (b) inert antibody (P3); (c) PNH I and PNH III cells; (d) discrete populations of PNH I, PNH II, and PNH III cells; (e) PNH I and PNH II cells; (f) nearly entirely PNH III cells.
The cells of intermediate abnormality (PNH II cells) were more difficult to demonstrate than in red cells. PNH II cells were not detected by anti-CD16, and the proportion of patients having PNH II granulocytes (demonstrated by anti-CD55 or anti-CD59) was less than that having PNH I erythrocytes.

Two techniques were used to prepare the granulocytes: the unseparated granulocyte technique, in which the red cells of whole blood were hemolyzed and the granulocytes reacted with the antibody and analyzed, and the separated granulocyte technique, in which the granulocytes were separated from other blood elements by differential centrifugation before testing. Whereas abnormal granulocytes could always be detected in the simpler unseparated assay, the distinctions between different populations and the proportions of cells in each population were less clear than in the tests using separated granulocytes. With both tests, a small number of "granulocytes" in normal blood did not stain with the antibody; as with the red cells, this was not consistent in a given patient and was thought to be artifactual. However, the number of cells in normal blood that did not bind the antibody was very much higher with the unseparated granulocyte technique; for this reason, that technique, although simpler, should be used with caution in the analysis of blood for the diagnosis of PNH, particularly when the proportion of abnormal cells is low. Samples older than 2 days could not be used for analysis because of poor quality of the cells.

In general, the proportion of abnormal granulocytes detected was greater than the number of abnormal red cells from the same blood sample. This observation has been previously found by flow cytometry and other techniques. The explanation lies in part in the fact that the survival in the circulation of the abnormal granulocytes appears to be normal, whereas that of the abnormal red cells is reduced. Thus, the percentage of circulating abnormal granulocytes more nearly represents the proportion of abnormal cells delivered to the circulation.

Cells with less than normal amounts of the GPI-linked proteins (PNH II cells) were demonstrated in both erythrocytes and granulocytes in a majority of cases when anti-
CD59 was used for detection. These cells were less easily seen with anti-CD55 and were not observed in granulocytes with anti-CD16. In some cases, the partial deficiency is due to a missense mutation of the pig-A gene\(^1\),\(^2\); in others, the identified defect appears to be one that would lead to a premature stop codon.\(^2\) The reason for this discrepancy is under investigation.

The deficiency of the GPI-dependent proteins could be demonstrated in the platelets of patients with PNH but the fluorescence of normal and PNH I platelets was different from the negative control and the distinction between PNH I and PNH III platelets was difficult and imprecise.

GPI-dependent protein deficient lymphocytes are demonstrable in most if not all patients with PNH\(^3\),\(^4\),\(^5\),\(^6\),\(^7\),\(^8\),\(^9\); immortalized lymphocyte cell lines have played an important role in the identification of the genetic defect in PNH.\(^10\)\(^\text{--}\)\(^12\) In most patients, the proportion of abnormal lymphocytes is small, particularly relative to the proportion of abnormal erythrocytes or granulocytes in the same blood. In some studies, the abnormal lymphocytes were missed entirely if they were in fact present.\(^11\) Thus, analysis of lymphocytes for GPI-linked proteins to make the diagnosis of PNH is not recommended. On the other hand, in patients who recover from PNH with disappearance of abnormal erythrocytes and granulocytes may have a persistent population of GPI-deficient lymphocytes.\(^12\) This is thought to be due to the longevity of lymphocytes compared with other blood cells.

Flow cytometry and MoAbs to GPI-dependent proteins have been used to analyze the cells of the bone marrow in PNH. In general, the proportion of abnormal cells corresponds roughly to that of the granulocytes. In exceptional cases, the abnormal cells in the bone marrow can be detected in the marrow for several months before they appear in the peripheral blood.\(^13\)

Small populations of cells deficient in GPI-dependent proteins can be demonstrated in the blood of 15% to 30% of patients with aplastic anemia, particularly after treatment with antithymocyte globulin.\(^14\)\(^\text{--}\)\(^16\) These populations are diagnostic of PNH by definition, but the clinical syndrome often remains that of aplastic anemia. These findings indicate a close but not clearly understood relationship between the two syndromes.\(^17\)\(^\text{--}\)\(^18\)

These studies clearly demonstrate the utility of MoAbs and flow cytometry in the diagnosis of paroxysmal nocturnal hemoglobinuria. Flow cytometry is shown in these studies to be superior to the CLS assay in that small populations of abnormal cells may be missed occasionally in the CLS test (1 patient in this series). Further, flow cytometry distinguishes much better the cells of intermediate abnormality (PNH II cells); while this may not be of great interest diagnostically, it is of major importance in characterizing the abnormalities in the cells of different patients.

We have previously shown that the CLS test was superior to either the Ham test or the sucrose lysis test.\(^19\) Even when the Ham test is done well, it does not detect small populations of abnormal cells (which results in a decrease in sensitivity), the degree of lysis does not accurately reflect the proportion of abnormal cells,\(^20\) and the degree of abnormality of the

abnormal cells cannot be assessed. Furthermore, the test is falsely positive in the rare disorder hereditary erythrocystic multinucularity with a positive acidified-serum lysis test (HEMPAS) congenital dyserythropoietic anemia, type II).\(^21\) The sucrose lysis test is technically easier to perform but has a higher rate of false-positive results\(^22\) (thus decreasing its specificity). Although it more accurately detects the proportion of PNH III red cells than the Ham test, it likewise does not delineate the abnormality of these cells and does not quantitatively delineate the number of PNH II cells.\(^23\)

Flow cytometry has, in addition, the advantage that the reagents can be standardized; the tests depending upon hemolysis use normal human serum as a reagent and there may be great variability in the potency of normal serum to lyse the abnormal red cells.\(^24\) Further, the reproducibility of the results from flow cytometry is greater than that of either other test. Red cells can be stored up to 3 weeks without change in the characteristics of the cells whereas granulocytes are difficult to analyze if they are more than 12 to 24 hours old. The estimated costs of this more accurate diagnostic technique may be 1 to 1.5 times as much as the conventional but less accurate serological tests.

Flow cytometry in the diagnosis of PNH is specific in that no other clinical syndrome is characterized by cells with the phenotype demonstrated by this technique. Hematopoietic cells congenitally deficient in CD55\(^46\),\(^47\) and CD59\(^48\) have been described, but these extremely rare conditions are readily distinguished from PNH by the facts that 100% of the cells are deficient in these syndromes and only one of the proteins is deficient. Because flow cytometry is able to demonstrate the characteristic cells with greater certainty and precision than previous methods using secondary effects (eg, increased sensitivity to complement lysis), we propose that the analysis by flow cytometry of erythrocytes using anti-CD59 or of granulocytes using either anti-CD59 or anti-CD55 be the standard diagnostic test for paroxysmal nocturnal hemoglobinuria.

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