Immunophenotypic Analysis of Reticulocytes in Paroxysmal Nocturnal Hemoglobinuria

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The hematologic disorder paroxysmal nocturnal hemoglobinuria (PNH) occurs following an acquired somatic mutation in the Piga gene within a bone marrow stem cell. The progeny of this mutated cell cannot synthesize glycosylphosphatidylinositol (GPI) anchors, with a resultant deficiency in surface expression of all GPI-linked proteins. The protean clinical manifestations of PNH presumably result from the deficiency of these GPI-linked surface proteins. To explain the observation that neutrophils are affected at a significantly higher percentage than circulating erythrocytes and to analyze the proliferative rates of erythroid production in PNH, we studied 25 patients using flow cytometry. The fluorescent dye thiazole orange was used to detect reticulocytes, and CD59 monoclonal antibody was used to identify GPI-deficient cells. In contrast to the mature circulating erythrocytes, the percentage of abnormal reticulocytes was similar to the percentage of affected neutrophils. However, the vast majority of reticulocytes was completely GPI-deficient, ie, were type III cells, even in patients with only modest numbers of circulating type III erythrocytes. In addition, greater than 5% type II reticulocytes were identified in only 3 patients, although greater than 5% type II mature erythrocytes were identified in 10 of 25 patients. The results show that the erythroid and neutrophil bone marrow precursors have an equivalent proliferative advantage in PNH. The data also have important implications for the origin of type-II erythrocytes in PNH.

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MATERIALS AND METHODS

Patients. Twenty-five patients with PNH were studied, including 16 females and 9 males. The patients ranged in age from 14 to 64 years (mean, 33 years). All of the patients had reticulocyte counts of more than 1.5%, and only 1 had received an erythrocyte transfusion within the previous 3 months. Controls consisted of 14 healthy adult volunteers.

Reagents. Retic-COUNT (thiazole orange; TO) was purchased from Becton Dickinson (San Jose, CA). The monoclonal antibody (MoAb) 10G10 is specific for CD59 and was the gift of Dr M. Telen (Durham, NC). Nonreactive MoAb F3 was used as a negative control. Fluorescein-conjugated goat F(ab')2, antimouse IgG (GAM-FITC) was purchased from Biosource International (Camarillo, CA). Phycoerythrin-conjugated rabbit F(ab')2, antimouse IgG (RAM-PE) was obtained from Zymed (South San Francisco, CA). Normal goat serum, normal rabbit serum, and human group AB serum were purchased from GIBCO-BRL (Grand Island, NY). All other chemicals were of reagent or chemical grade. The buffer used for washing of cells was veronal-buffered saline (GVB-EDTA), which was composed of 5.0 mmol/L sodium barbital, 0.15 mol/L NaCl, 0.1% gelatin, and 0.015 mol/L EDTA (pH 7.5).

Preparation of erythrocytes and neutrophils. Venous PB was drawn from 25 PNH patients and 14 normal controls into EDTA-containing tubes. After centrifugation (1,500 rpm for 5 minutes) and 2 washes with GVB-EDTA, the packed cells were resuspended to 1 × 10^7/mL for immunophenotypic analysis. Immune phenotype analysis. Single-color flow analysis was performed using a two-step immunophenotype assay. A total of 100 μL of washed cells were incubated with 100 μL 10G10 or F3 (1:500 dilution ascites) for 30 minutes at room temperature (RT). 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 for another 30-minute incubation at RT, followed by 2 washes in GVB-EDTA. The pelleted cells were resuspended in 1.0 mL buffer and analyzed on an ORTHO-CYTORON (Ortho Diagnostics, Raritan, NJ). Erythrocytes and neutrophils were easily distinguished using side scatter and forward scatter, allowing analysis of both types of cells.

Two-color analysis was performed as above, except that RAM-PE was used as the developing antibody after incubation with 10G10 or F3. The cell pellet was resuspended in 1.0 mL TO and was
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...and analyzed was significantly higher than the number of affected erythrocytes, 65.5% reticulocytes in PNH patients. Blood from 25 PNH patients was purified and reported representing the mean value. For circulating neutrophils, the average number of affected cells was not significantly different from the number of GPI-deficient reticulocytes, as depicted in the Materials and Methods section and reported as the percentage of CD59- cells. The result for each patient is represented by a diamond (.), with the horizontal line representing the mean value. For circulating neutrophils, the average number of affected cells was 81.8% ± 3.8% (mean ± SEM), which was significantly higher than the number of affected erythrocytes, 65.5% ± 5.4% (P = .016). In contrast, the number of GPI-deficient neutrophils was not significantly different from the number of GPI-deficient reticulocytes, 89.8% ± 1.9% (P > .05).

Enumeration and comparison of GPI-deficient erythrocytes and neutrophils. Using immunophenotypic analysis of venous PB, the percentage of circulating CD59- (GPI-deficient) neutrophils and erythrocytes was quantitated in 25 PNH patients. Both partially deficient (type II) cells and completely deficient (type III) cells were identified in some patients, and the total number of these cells was used for statistical comparison.

As a group, the PNH patients had a mean of 34.5% type I erythrocytes, 6.1% type II erythrocytes, and 59.4% type III erythrocytes. The normal volunteers had 100% normal (type I) erythrocytes. Figure 1 shows that the percentage of affected neutrophils (81.8% ± 3.6% [mean ± SEM]) was significantly higher than the number of PNH erythrocytes (65.5% ± 5.4%; P = .016).

Analysis of reticulocytes in PNH patients. Although the percentage of CD59- erythrocytes was significantly less than the percentage of affected neutrophils, we realized that ongoing in vivo hemolysis might lead to an accelerated loss of PNH erythrocytes and an artificially low number of circulating cells. To quantitate the rate of production of GPI-deficient erythroid cells more accurately, we next analyzed reticulocytes using two-color immunophenotype analysis. For our population of 25 PNH patients, the mean percentage of circulating reticulocytes was 3.9% (range, 1.6% to 7.2%). Figure 1 also shows the percentage of GPI-deficient reticulocytes and shows that the average number of PNH reticulocytes was significantly greater than the number of PNH erythrocytes (P < .001) but was not significantly different from the number of PNH neutrophils.

Figure 2 shows representative examples of flow cytometry histograms for 3 patients (referred to by unique patient number [UPN]) and a control volunteer. The data include both the single-color histogram for total erythrocytes (FL1 indicates that CD59 was used for identification of GPI-linked protein expression) and the double-color histogram for reticulocytes (TO indicates that thiazole orange was used for detection of reticulocytes; FL2 indicates that CD59 was incubated for 60 minutes at RT in the dark. The cells were then immediately analyzed on the ORTHO-CYTORON, using color compensation for cross-over of fluorescence signals between the two detectors.

Statistics. Statistical analysis was performed using the Primer of Biostatistics package (McGraw-Hill, New York, NY). Statistical comparisons were performed using the Student's t-test and the Wilcoxon signed rank test.

RESULTS

Enumeration and comparison of GPI-deficient erythrocytes and neutrophils. Using immunophenotypic analysis of venous PB, the percentage of circulating CD59- (GPI-deficient) neutrophils and erythrocytes was quantitated in 25 PNH patients. Both partially deficient (type II) cells and completely deficient (type III) cells were identified in some patients, and the total number of these cells was used for statistical comparison.

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and accurate method of analyzing this abnormal cell population in PNH patients. TO is a 488-nm–excitable fluorescent dye that is well-suited for flow cytometry analysis and correlates well with manual determination of reticulocyte percentage. The use of TO in two-color analysis of reticulocytes has been previously reported in conjunction with MoAbs against the transferrin receptor or glycoporphin-A. We modified this approach using the CD59 MoAb 10G10 to identify and quantitate the various abnormal reticulocyte populations in PNH.

In each patient, the vast majority of reticulocytes were type III cells, ie, completely GPI-deficient. An average of 86.5% of the reticulocytes were type III cells, whereas only 3.3% were type II, and 10.1% ± 1.9% of the reticulocytes had normal CD59 expression.

Analysis of the reticulocytes in all 25 PNH patients showed that, in each case, the vast majority of reticulocytes had no expression of CD59, even in patients with sizable proportions of circulating type II erythrocytes. Figure 3 shows the percentage of reticulocytes according to their level of CD59 expression for all 25 patients. An average of 86.5% ± 2.1% (mean ± SEM) of the reticulocytes were type III cells, whereas only 3.3% ± 1.3% were type II, and 10.1% ± 1.9% of the reticulocytes had normal CD59 expression. Only 3 of the patients had greater than 5% type II reticulocytes.

**DISCUSSION**

Despite numerous reports describing flow cytometry results on PB cells in PNH, there have been no prior studies focusing on reticulocytes in this disorder. Analysis of PNH reticulocytes is important, however, because most patients have elevated numbers of reticulocytes that represent more closely the recent erythroid output of the BM. In contrast, circulating mature erythrocytes are subject to complement-mediated intravascular lysis; therefore, the percentage of abnormal erythrocytes may not accurately reflect the proliferative rates of normal and abnormal erythroid progenitor cells.

We studied 25 PNH patients with evidence of active erythropoiesis (reticulocyte count, >1.5%) who had not received recent erythrocyte transfusions. Immunophenotypic analysis of reticulocytes using two-color flow cytometry was an easy
are type III cells and, therefore, would manifest these PNH characteristics to a greater extent than the total erythrocyte population.

REFERENCES


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