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 were 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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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.

As a group, the PNH patients had a mean of 34.5% type I erythrocytes, 61.6% 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 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).

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
used). In Fig 2A, UPN 210 had only type I and type III erythrocytes detected; Fig 2B shows that UPN 296 had type I, II, and III erythrocytes present; and Fig 2C shows that UPN 247 had only type II and III erythrocytes identified. In each case, however, the reticulocytes were almost exclusively in the type III population. The normal volunteer (Fig 2D) had only type I cells identified.

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, ie, had no CD59 surface expression. Only 3.3% ± 1.3% of the reticulocytes were type II cells, 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,13-17 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 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 and accurate method of analyzing this abnormal cell population in PNH patients. TO is a 488-nm-exciteable fluorescent dye that is well-suited for flow cytometry analysis and correlates well with manual determination of reticulocyte percentage.18 The use of TO in two-color analysis of reticulocytes has been previously reported in conjunction with MoAbs against the transferrin receptor19 or glycoporphin-A.20 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 (Fig 3). As a group, the 25 patients had 59.4% type III erythrocytes, but 86.5% of the reticulocytes were type III cells. This percentage of abnormal reticulocytes was similar to the percentage of abnormal circulating neutrophils, providing evidence that the proliferative rates of the erythroid and neutrophils BM precursors are probably equivalent in PNH. Furthermore, these findings suggest that premature peripheral destruction of PNH erythrocytes may account fully for the observation that most patients have significantly more GPI-deficient neutrophils than mature erythrocytes. Without GPI-linked surface proteins, PNH erythrocytes cannot remove membrane-bound complement or vesiculate properly,21 rendering them susceptible to intravascular lysis.

Because they show a paradox between the percentages of type II reticulocytes and type II mature erythrocytes, these data are also important for the understanding of the origin of type II cells in PNH. Only 3 of the 25 patients had greater than 5% type II reticulocytes (6%, 15%, and 30%, respectively), although a total of 10 patients had greater than 5% mature type II erythrocytes. It is reasonable to believe that type II reticulocytes can mature into type II erythrocytes, with a near-normal in vivo lifespan. However, type II erythrocytes were also detected in 7 patients with virtually no type II reticulocytes (for examples see Figs 2B and C). We currently do not have an explanation for all of these observations but propose a series of testable hypotheses. (1) For patients with measurable type II reticulocytes, the abnormal PNH clone has a missense Piga mutation, with partial GPI anchor formation and dim surface expression of GPI-linked proteins (see Ware et al22 for an example); (2) for patients with sizable populations of both type II and type III reticulocytes, there are 2 independent PNH clones within the BM, one with a missense Piga mutation and the other with a Piga mutation leading to a complete loss of function (Bessler et al22 and our unpublished observations); and (3) in patients with type II erythrocytes but no evidence of type II reticulocytes, the surface expression of GPI-linked proteins derives either from the loss of expression on type I cells or from the gain of expression on type III cells, eg, from plasma adsorption.

Finally, these data help explain laboratory observations first reported almost 40 years ago. Several investigators noted that, when PNH erythrocytes were separated by density centrifugation, the upper portion (reticulocyte-rich) consistently had less acetylcholinesterase activity23,24 or more sensitivity to acidified serum or complement-mediated lysis.24,25 Our data show that virtually all PNH reticulocytes...
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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