CONCISE REPORT

Paroxysmal Nocturnal Hemoglobinuria Erythrocytes Are of Two Distinct Types: Positive or Negative for Acetylcholinesterase

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Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired disorder. Erythrocytes isolated from PNH patients show increased sensitivity to complement and decreased acetylcholinesterase (AChE) activity. In this study, indirect immunofluorescence analysis of a monoclonal antibody specific for a surface epitope of human erythrocyte AChE is used to quantitate the content of this enzyme at the single-cell level. Flow-cytofluorimetric analysis of erythrocytes from normal donors indicates that all erythrocytes contain detectable levels of the surface epitope with a strong correlation between cell size and enzyme content. In contrast, erythrocytes from PNH patients show two distinct populations of erythrocytes; namely, those containing a normal content of AChE and a second population containing no detectable AChE. The AChE-negative population of cells is quantitatively complement-sensitive. These data support suggestions that PNH is a clonal disorder resulting in two distinct types of circulating erythrocytes. The abnormal clone produces cells that are both surface-AChE-negative and complement-sensitive. In addition, the method described provides an attractive alternative for the diagnosis and quantitative evaluation of abnormal erythrocytes in PNH patients.

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MATERIALS AND METHODS

Immunofluorescent labeling of erythrocytes. Whole blood was drawn from normal subjects and PNH patients with EDTA used as an anticoagulant. A 200-μL volume of this blood was washed three times with 500 μL of isotonic phosphate-buffered saline (PBS), pH 7.4. The cells were resuspended in 500 μL of PBS, and 10 μL of AE-2 monoclonal antibody ascites fluid (produced from AE-2 mouse

Fig 1. Flow-cytofluorimetric analysis of acetylcholinesterase content on erythrocytes can be used to detect PNH erythrocytes in a PNH patient. Samples were stained immunofluorescently for surface acetylcholinesterase (green fluorescence) relative cell size (light scatter) or cell number is shown. (A) Erythrocytes of a normal individual showing all cells staining positive for acetylcholinesterase. (B) Erythrocytes of a PNH patient demonstrating that a subpopulation of erythrocytes from this patient lack the acetylcholinesterase antigen. (C) and (D) Controls for normal and PNH erythrocytes, respectively, where monoclonal antibody AE-2 was omitted from the labeling procedure.

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Panel data are shown replotted in the bottom cells for the four levels of increasing density between cell size and AChE content. Contour levels shown in the erythrocytes from a normal donor indicate a positive correlation of fluorescence for each light-scatter channel.

**Fig 2.** Plots of acetylcholinesterase labeling vs light scattering of erythrocytes from a normal donor indicate a positive correlation between cell size and AChE content. Contour levels shown in the top panel are set at 5–20, 21–50, 51–150, and greater than 150 cells for the four levels of increasing density in the plot. The same data are shown replotted in the bottom panel as the mean channel of fluorescence for each light-scatter channel.

hybridoma cell line obtained from the American Type Culture Collection, ATCC No. HB-73) was added to the cells. This concentration of ascites fluid was determined to be a saturating dose of primary antibody as determined by separate titration studies. The suspension was allowed to incubate at room temperature for 20 minutes and subsequently washed twice with 500 µL of PBS to remove unbound antibody. Indirect immunofluorescence of the bound immunoglobulin was accomplished by subsequently incubating the erythrocytes with saturating doses (1:100 PBS dilutions) of fluorescein-conjugated goat antimouse IgG (whole molecule, 2.3 mg IgG/mL, F/P molar ratio 4.1) and fluorescein-conjugated rabbit anti-goat IgG (whole molecule, 2.7 mg IgG/mL, F/P molar ratio 4.1) (Sigma Chemicals, Inc) for 20 minutes each with two PBS washes after each incubation. Control samples were treated in an identical manner except the first monoclonal antibody was omitted from the labeling protocol. Similar control results were obtained using ascites fluid containing monoclonal antibodies directed against irrelevant proteins yeast cytochrome c oxidase and platelet membrane glycoprotein IIb (data not shown). Where indicated, complement-sensitive cells were first lysed by the sucrose hemolysis test before immunoglobulin labeling.

**Flow cytometry.** Labeled erythrocytes were diluted approximately 1:1,000 in PBS immediately before flow-cytometric analysis. All stained cells were analyzed at approximately 500 cells/s on an EPICS-V flow cytometer (Coulter Electronics, Inc, Hialeah, Fla) after excitation with a Spectra-Physics 164-05 argon laser, adjusted to deliver 800 mW at 488 nm. Low-angle light scattering was detected through a 2.0 neutral density filter, and green fluorescence was detected at right angles to the incident laser beam through a 510-nm interference and 515-nm bandpass filter combination on the green photomultiplier of the instrument. The light-scatter and fluorescence signals from each cell are collected and stored by the Coulter MDCADS integrated microcomputer analysis system in a 64 × 64 matrix array. Routinely, the fluorescence signals measured are gated on a light-scatter profile that precludes contributions from small cell debris and dust. Data collection was set up to stop after 100,000 cells have been analyzed.

**RESULTS**

Erythrocytes isolated from normal donors and PNH patients were analyzed by flow cytometry after indirect immunofluorescence labeling of surface AChE with the monoclonal antibody AE-2. Figure 1 displays the three-parameter histogram of light scatter, green fluorescence, and cell number for these cells. Normal erythrocytes all could be shown to have detectable levels of AChE on their surface, as indicated by their positive green fluorescence signal. Figure 2 is a scattergram of the same normal cell sample shown in Fig 1. This alternative presentation of the data shows that in normal individuals, the mean green fluorescence signal increases with increasing light-scatter signal, which indicates a higher level of AChE on the surface of the larger erythrocytes. In contrast, erythrocytes from PNH patients showed two distinct populations of cells with respect to AChE content (Fig 1). The first population has an AChE content and distribution similar to those of the normal erythrocytes. The second population of erythrocytes has undetectable levels of AChE on their surfaces.

Figure 3 shows that cells that are complement-sensitive in the PNH patient lack AChE. Erythrocytes from a normal donor and a PNH patient were independently analyzed for their percentage of PNH cells by complement lysis. The complement lysis test indicated that 19.0% of the hemoglobin in the PNH sample was released. This number was very close to the 19.3% of the erythrocytes detected as AChE-negative by integration of the number of cells under the AChE-negative peak of the PNH cells in Fig 3. There was no hemoglobin release from the normal erythrocytes, and no AChE-negative cells were detected by flow cytometry. This result suggests that the two analyses were measuring the same cell population. To test this contention further, the remaining PNH (the cells insensitive to complement lysis) were spun down, labeled for AChE, and analyzed by flow cytometry. Figure 3 clearly indicates that the PNH cells that are complement-lysed lack the surface enzyme...
complement pathway is involved in the anemia. Accordingly, glycoprotein (PAS I C3 convertase activity. The PNH cells bind the 3Cb covalently on the surface of PNH cells has been attributed to abnormalities on the PNH erythrocyte membrane that favor increased sensitivity to that of normal individuals. This fact, in combination with changes in the red cell membrane that ultimately make the cell more complement-sensitive than normal cells. Reports suggest that both normal and PNH erythrocytes assemble the alternative pathway C3 convertase in the same fashion. However, the PNH cells activate more C3, cytocens in PNH is currently under active investigation. Another erythrocyte membrane component, protein 3, and another component, protein 4, also bind 3Cb. The PNH cells bind the 3Cb covalently to the hydroxyl group of the major erythrocyte sialoglycoprotein (PAS 1, glycoporphin A). Evidence has also been presented that the sialoglycoproteins of PNH erythrocytes themselves be altered in some PNH patients. Other investigators have suggested that an inhibitor that is involved in regulating the alternate pathway is absent in a subpopulation of PNH erythrocytes. It is clear from these studies that considerable variability may exist among PNH patients with respect to changes in the red cell membrane that ultimately make the cell more complement-sensitive. Accordingly, a current PNH cell classification scheme classifies PNH patients according to their degree of complement sensitivity. In some PNH patients, the complement sensitivity is very close to that of normal individuals. This fact, in combination with the variability of complement potency of donor sera used for hemolysis tests and the low percentage of complement-sensitive cells in some PNH patients, makes positive diagnosis of PNH difficult in some patients.

In this study, we have shown that the ectoenzyme acetylcholinesterase is absent in the complement-sensitive clones of cells present in a PNH patient. Although the enzyme does not appear to play any role in the disease state, it may present a clearer criterion for the diseased cells than complement sensitivity. We have therefore developed a method for the detection of acetylcholinesterase at the single-cell level. To date, over 50 blood samples from seven previously diagnosed PNH patients and over 100 normal samples have been analyzed by this method. In all PNH cases, a distinct population of AChE-negative cells is discernible in addition to cells with the normal content of AChE. All normal samples have lacked this AChE-negative population of cells. This method has distinct advantages over current PNH diagnostic procedures: It is quantitative for the percentage of abnormal cells circulating in the PNH patient, it uses a monoclonal antibody that is readily available as a standardized diagnostic reagent, and it requires a very small blood volume, and it is capable of highly accurate and reproducible determinations even on samples with very low percentages of abnormal cells. Although it does require a flow cytofluorimeter for quantitative measurements, qualitative analyses can be performed on a fluorescence microscope. Finally, the method allows us not only to determine quantitatively the percentage of abnormal erythrocytes but also to correlate the acetylcholinesterase content of a cell with any other parameter detectable by flow cytofluorimetry. In this study, we have also noted a positive correlation between erythrocyte size and acetylcholinesterase content. This is in contrast to previous studies, which indicate that there is no correlation between another erythrocyte membrane component, protein 3, and cell size. Such results can be explained by proposing a selective loss of membrane material during erythrocyte aging. The potential of multiparameter analysis of erythrocytes in PNH is currently under active investigation.

REFERENCES

acetylcholinesterase. Thus, erythrocytes detected as abnormal because they lack surface AChE in PNH individuals are the same set of cells that are complement-sensitive.

DISCUSSION
Current evidence suggests that in PNH, an altered complement pathway is involved in the anemia. Accordingly, much of the recent work on this disease has focused on why the PNH cell is more complement-sensitive than normal cells. Reports suggest that both normal and PNH erythrocytes activate more C3, resulting in the deposition of increased levels of C3b on the cell surface. Since a similar number of activated C3b binds to normal and PNH cells, the greater fixation of C3b on the surface of PNH cells has been attributed to abnormalities on the PNH erythrocyte membrane that favor increased C3 convertase activity. The PNH cells bind the 3Cb covalently to the hydroxyl group of the major erythrocyte sialoglycoprotein (PAS 1, glycoporphin A). Evidence has also been presented that the sialoglycoproteins of PNH erythrocytes may themselves be altered in some PNH patients. Other investigators have suggested that an inhibitor that is involved in regulating the alternate pathway is absent in a subpopulation of PNH erythrocytes.

It is clear from these diverse studies that considerable variability may exist among PNH patients with respect to changes in the red cell membrane that ultimately make the cell more complement-sensitive. Accordingly, a current PNH cell classification scheme classifies PNH patients according to their degree of complement sensitivity. In some PNH patients, the complement sensitivity is very close to that of normal individuals. This fact, in combination with the variability of complement potency of donor sera used for hemolysis tests and the low percentage of complement-sensitive cells in some PNH patients, makes positive diagnosis of PNH difficult in some patients.

In this study, we have shown that the ectoenzyme acetylcholinesterase is absent in the complement-sensitive clones of cells present in a PNH patient. Although the enzyme does not appear to play any role in the disease state, it may present a clearer criterion for the diseased cells than complement sensitivity. We have therefore developed a method for the detection of acetylcholinesterase at the single-cell level. To date, over 50 blood samples from seven previously diagnosed PNH patients and over 100 normal samples have been analyzed by this method. In all PNH cases, a distinct population of AChE-negative cells is discernible in addition to cells with the normal content of AChE. All normal samples have lacked this AChE-negative population of cells. This method has distinct advantages over current PNH diagnosis procedures: It is quantitative for the percentage of abnormal cells circulating in the PNH patient, it uses a monoclonal antibody that is readily available as a standardized diagnostic reagent, and it requires a very small blood volume, and it is capable of highly accurate and reproducible determinations even on samples with very low percentages of abnormal cells. Although it does require a flow cytofluorimeter for quantitative measurements, qualitative analyses can be performed on a fluorescence microscope. Finally, the method allows us not only to determine quantitatively the percentage of abnormal erythrocytes but also to correlate the acetylcholinesterase content of a cell with any other parameter detectable by flow cytofluorimetry. In this study, we have also noted a positive correlation between erythrocyte size and acetylcholinesterase content. This is in contrast to previous studies, which indicate that there is no correlation between another erythrocyte membrane component, protein 3, and cell size. Such results can be explained by proposing a selective loss of membrane material during erythrocyte aging. The potential of multiparameter analysis of erythrocytes in PNH is currently under active investigation.
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