**Separation of the Acetylcholinesterase-Deficient Red Cells in Paroxysmal Nocturnal Hemoglobinuria**

By Feng-Lan Chow, Sharon E. Hall, Wendell F. Rosse, and Marilyn J. Telen

Blood of patients with paroxysmal nocturnal hemoglobinuria (PNH) most often contains two or more populations of erythrocytes—one population with normal sensitivity to lysis by complement (PNH I cells) and a second population of moderately abnormal cells (PNH II cells) or markedly abnormal cells (PNH III cells). PNH II and III cells exhibit moderately and markedly increased sensitivity to lysis by complement, respectively, as well as other membrane defects. We have devised a method for isolating pure.

Paroxysmal nocturnal hemoglobinuria (PNH) is characterized by red blood cells that are abnormally sensitive to the hemolytic action of complement. This abnormality is usually present in some but not all red cells; thus, populations of red cells with different sensitivity to the hemolytic action of complement are present in the circulation. Three degrees of sensitivity to complement have been described: some of the red cells may be normal (PNH I cells), some may be moderately (two- to fourfold) more sensitive than normal (PNH II cells), and some may be markedly (15- to 25-fold) more sensitive than normal (PNH III cells). These populations occur in various combinations and various proportions in different patients with PNH. It is this mixture of cells that has made it difficult to analyze the membrane defect leading to increased lysis by complement.

In 1960, Auditore et al found that the activity of the enzyme acetylcholinesterase was variably deficient on the red blood cells of patients with PNH. Kunstling and Rosse demonstrated that it was the cells that were abnormally sensitive to the lytic action of complement that were deficient; the normal PNH I cells appeared to have normal amounts of the enzyme. Recently, Chow et al demonstrated by the use of monoclonal antibodies to the enzyme that the protein immunologically characteristic of the enzyme was lacking in these cells. In the present paper, we demonstrate how these facts have been used to devise a technique to separate the complement-sensitive from the complement-insensitive red cells in the blood of patients with PNH. We have used this technique to define a fourth variant population in PNH red blood cells, a subpopulation of PNH III cells that are slightly less sensitive to complement lysis than the majority of PNH III cells.

**MATERIALS AND METHODS**

*Red blood cells.* Blood from normal donors or patients with PNH was stored under sterile conditions in Alsever's solution. At the time of use, the erythrocytes were washed in phosphate-buffered saline (PBS), pH 7.4, and adjusted to a concentration of 2 x 10^8/mL.

*Antibodies.* Monoclonal antibodies (AE-1, AE-2, AE-3, and AE-4) made by mouse hybridomas against four different epitopes of the acetylcholinesterase molecule were the kind gift of Dr D. Fambrough in the form of purified IgG. In addition, AE-1 and AE-2 hybridoma cells were obtained from American Type Culture Collection and grown as ascites tumors in BALB/c mice. P3 (P3x63/Ag8), the immunoglobulin product of a murine myeloma cell line, was also grown as murine ascites tumors and used as an isotype specific (IgG1) control for the reactions of the AE monoclonal antibodies.

Antibody to mouse IgG made in rabbits was obtained as affinity-purified IgG from Cappel Laboratories, Malvern, Pa. This was shown to react with monoclonal antibody attached to red blood cells by binding of radioiodinated staphylococcal protein A (SPA).

*Complement lysis sensitivity tests.* The sensitivity of human red cells to the lytic action of complement was determined by a modification of the complement lysis sensitivity (CLLS) test of Rosse and Dacie. The total reaction volume was reduced to 375 µL, but the proportions and materials remained the same. The value used to compare sensitivity of different cell populations to lysis by complement is the CLS H50, defined as the reciprocal of the dilution of serum used (270 in these studies) divided by the milliliter equivalents of serum required to obtain lysis of 50% of the cells.

*Binding of monoclonal antibodies to normal red cells.* To determine which monoclonal antibodies best bind to the acetylcholinesterase on normal cells, equal volumes (100 µL) of a 5% suspension of washed normal red blood cells and a saturating dilution of monoclonal antibody (or a comparable dilution of control ascitic protein [P3]) were incubated at room temperature for 30 minutes. The cells were then washed twice, brought to a volume of 100 µL, and incubated with an equal volume of rabbit antimouse IgG (2 mg/mL diluted 1:50) at room temperature for 30 minutes. After washing, the cells were again suspended in 100 µL and incubated with 100 µL of [125I]-labeled SPA (8 x 10^6 cpm). Triplicate aliquots of 50 µL each were layered on a mixture of phthalate esters (1.5 parts N-butyphthalate, Fisher Scientific Co, Pittsburgh, Pa to one part bis-2-ethylhexyl phthalate, Eastman Kodak Inc, Rochester, NY) in plastic microcentrifuge tubes and centrifuged in a Beckman microfuge (Beckman Instruments, Fullerton, Calif). The cells were removed by clamping and cutting the tube, and the radioactivity was counted in a well-type scintillation counter.

*Separation of red blood cells on a column.* Washed red cells (1 x 10^8/mL) were incubated with equal volumes of each monoclonal antibody (AE-1 and AE-2) at a dilution that saturated all...
sites for 30 minutes at room temperature on a rocker platform. They were washed by centrifugation, suspended to the original volume, and incubated with an equal volume of rabbit antimouse IgG (1:50) for 30 minutes at room temperature on a rocker. After two washes, they were pipetted onto the top of the separation column.

The separation column consisted of 10 mL of Sepharose 6MB to which SPA had been covalently attached (obtained from Pharmacia Fine Chemicals, Piscataway, NJ). The column was packed by gravity and washed thoroughly with PBS buffer containing 1% bovine serum albumin before each use.

After application of the cells, the flow rate was adjusted to about 0.2 mL/min; this flow rate was maintained until no further cells could be eluted from the column (usually about 50 minutes). The flow rate was then increased to 3 to 4 mL/min again until no further cells were eluted.

The column was regenerated by thorough (ten-bed volume equivalents) washing with 0.1 mol/L glycine-HCl buffer, pH 3, containing 0.1 mol/L NaCl. It was then thoroughly washed with PBS and stored at 4°C in 0.1 mol/L phosphate buffer with sodium azide (0.05% w/v) pH 6.0.

RESULTS

Binding studies. As shown in Table 1, the monoclonal antibodies AE-1, AE-2, and AE-3 all bound via rabbit antimouse IgG about the same amount of radiolabeled SPA to red cells; AE-4 bound less under similar conditions. Studies using AE-2 monoclonal antibody and radiolabeled F(ab')2 antimouse IgG (Amersham Corp, Arlington Heights, Ill) showed that maximum binding of the radiolabeled anti-IgG was ten to 50 molecules per cell, indicating an extremely low density of antigen. No great increase in binding of radiolabeled SPA was found with combinations of monoclonal antibodies except in the case of the mixture of AE-1 and AE-2. For this reason, this combination was used for the chromatographic separation experiments.

Chromatography of normal red blood cells. When normal red blood cells sensitized with AE-1 and AE-2 and rabbit antimouse were chromatographed on the Sepharose 6MB-SPA column, no cells were eluted during the first (slow flow) phase; 80% ± 5% of the cells were eluted during the rapid flow phase. Cells not sensitized or sensitized with P3 and treated in the same way were eluted during the first (slow) phase (80% ± 10%). This indicated that red blood cells that had the acetylcholinesterase enzyme and that had been reacted with the antiacetylcholinesterase monoclonal antibodies were specifically retained on the column under these conditions.

To test the ability of the column to separate antibody-coated cells from those not coated by antibody, type A red cells from a normal donor were coated with AE-1 plus AE-2 and rabbit antimouse IgG; type O cells from a normal donor were reacted with the control protein, P3. The cells were

<table>
<thead>
<tr>
<th>Antibody*</th>
<th>125I-Labeled SPA (cpm Bound)</th>
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</thead>
<tbody>
<tr>
<td>AE-1</td>
<td>5,491</td>
</tr>
<tr>
<td>AE-2</td>
<td>5,777</td>
</tr>
<tr>
<td>AE-3</td>
<td>5,115</td>
</tr>
<tr>
<td>AE-4</td>
<td>1,254</td>
</tr>
<tr>
<td>AE-1 + AE-2</td>
<td>7,319</td>
</tr>
<tr>
<td>AE-1 + AE-3</td>
<td>6,612</td>
</tr>
<tr>
<td>AE-1 + AE-4</td>
<td>6,077</td>
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<tr>
<td>AE-2 + AE-3</td>
<td>6,557</td>
</tr>
<tr>
<td>AE-2 + AE-4</td>
<td>6,037</td>
</tr>
<tr>
<td>AE-3 + AE-4</td>
<td>6,098</td>
</tr>
<tr>
<td>P3 control</td>
<td>389</td>
</tr>
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</table>

*All antibodies used in saturating amounts, as determined by binding of radiolabeled antimouse IgG.

Fig 1. Separation of the populations of PNH cells by affinity chromatography. After incubation with monoclonal antibodies to acetylcholinesterase and rabbit antimouse antibody, PNH red cells were applied to a column containing Sepharose 6MB to which SPA was covalently attached. The PNH I cells bearing acetylcholinesterase were retarded (upper band), whereas those lacking the enzyme (PNH III cells) passed through the column (lower band).
mixed and chromatographed through the column. Two separate
populations were evident during the slow elution phase,
with only type O cells eluted during this phase as shown by
lack of agglutination of the eluted cells with anti-A. The type
A cells were eluted during the phase of rapid flow.

Chromatography of PNH red blood cells. When PNH
red blood cells of the common phenotype (PNH I and PNH
III cells) were treated in this way, there was a separation
on the column into two distinct populations (Fig 1): one
population eluted from the column during the first (slow)
phase of the elution process, the other eluted during the rapid elution
phase. When these separated cells were tested in the CLS
test, those that were not adherent to the column were entirely
PNH III cells (Fig 2, line B). Those that were adherent were
a mixture of the two populations but were enriched for PNH
I cells (Fig 2, curve C). If the cells retained on the column and eluted with rapid flow were again passed through the column, they were further enriched for PNH I cells (Fig 2, curve D), indicating that the contaminating PNH III cells did not remain on the column because of a specific reaction with the column.

Table 2. Complement Sensitivity of Affinity-Purified PNH III
Red Blood Cells

<table>
<thead>
<tr>
<th>Patient</th>
<th>Whole Blood Percentage</th>
<th>PNH III in</th>
<th>CLS</th>
<th>Percentage</th>
<th>H50 Units</th>
<th>PNH IIIb in</th>
<th>CLS</th>
<th>Percentage</th>
<th>H50 Units</th>
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</thead>
<tbody>
<tr>
<td>V.D.</td>
<td>73</td>
<td>65</td>
<td>36</td>
<td>35</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J.P.</td>
<td>58</td>
<td>65</td>
<td>31</td>
<td>35</td>
<td>17</td>
<td></td>
<td></td>
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<tr>
<td>W.M.</td>
<td>28</td>
<td>49</td>
<td>67</td>
<td>51</td>
<td>18</td>
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<td></td>
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</tr>
<tr>
<td>J.G.</td>
<td>44</td>
<td>69</td>
<td>38</td>
<td>31</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>O.P.</td>
<td>42</td>
<td>100</td>
<td>27</td>
<td>10</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E.D.</td>
<td>47</td>
<td>100</td>
<td>24</td>
<td>24</td>
<td>10</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>J.B.</td>
<td>37</td>
<td>100</td>
<td>37</td>
<td>37</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E.G.</td>
<td>61</td>
<td>100</td>
<td>41</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>A.H.</td>
<td>15</td>
<td>100</td>
<td>41</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>M.F.</td>
<td>60</td>
<td>100</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C.K.</td>
<td>&gt;95</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&gt;95</td>
<td>16</td>
</tr>
</tbody>
</table>

*CLS H50 is quantitated by determining the reciprocal of the dilution of
serum containing a normal amount of complement, 1 mL of which would
lyse 50% of the sensitized cells of a population in a total volume of 7.5
mL. Normal CLS is 2.0 to 3.2 CLS H50 units.

When cells containing PNH I and PNH II cells were
treated as previously described and passed through the
column, the PNH II cells were also eluted during the slow elution phase. The cells that were eluted during the slow elution phase were pure PNH II cells (Fig 3).

The use of the column to define a subpopulation of PNH
III cells. The red cells of 11 patients who were defined as
having PNH I and PNH III cells by conventional CLS
testing were separated on the column by the aforementioned
procedure. When the red cells lacking acetylcholinesterase
were tested in the CLS test, six of 11 were found to be of a
single population in that the analysis demonstrated a single
straight line (as in curve B, Fig 2). However, in four of 11
patients, there appeared to be a second population of cells
that were slightly less sensitive than the PNH III cells as
previously defined (Table 2 and Fig 4). The cells of one
patient appeared to consist entirely of these slightly less
sensitive cells (Table 2). When the PNH III cells from these
four patients were isolated by affinity chromatography and
lysed by complement activated in the fluid phase by cobra
venom factor, 100% of the PNH III cells from each patient
were lysed. Thus, the slightly less complement-sensitive

Fig 2. Analysis of the sensitivity to complement lysis of PNH I
and PNH III cells separated by affinity chromatography. The
analysis of CLS of the unseparated cells is shown in curve A: 50% of the cells were PNH I, and 50% were PNH III. The analysis of the CLS of the cells eluted from the column by slow flow rate is shown in curve B; these cells were entirely PNH III cells since the CLS curve forms a straight line. Results of the CIS of the cells are shown in curve C; the proportion of PNH I cells was increased from 50% to 97.1
%.

When these cells were rechromatographed, the proportion of PNH
II cells in the retained cell population increased to 97.1
%. As shown by curve D, indicating that the contaminating PNH III cells
did not remain on the column because of a specific reaction with the column.

Fig 3. Analysis of the sensitivity to complement lysis of PNH I
and PNH II cells separated by affinity chromatography. The analysis
of CLS of the unseparated cells is shown in solid symbols: 66%
of the cells were PNH I, and 34% were PNH II. The analysis of CLS
of the cells eluted from the column by slow flow rate is shown in
open symbols; these cells were entirely PNH II cells. The CLS of the
PNH II cells was not changed by passage through the column.
Lysis of 50% of the purified PNH II cells occurred at approximately
29-mL equivalents of serum, whereas lysis of 50% of the PNH II
cells within the unseparated cell suspension occurred at approxi-
mately 35-mL equivalents of serum.

Fig 4. Analysis of the sensitivity to complement lysis of PNH I,
PNH III, and PNH IIIb cells separated by affinity chromatography.
The analysis of CLS of the unseparated cells is shown in solid
symbols: 50% of the cells were PNH I, and 50% were PNH IIIa +
PNH IIIb. The analysis of CLS of the cells eluted from the column by
slow flow rate is shown in open symbols; 43% of these cells were
PNH IIIa cells, and 57% were PNH IIIb cells.
PNH III cells did not resemble PNH II cells in this respect. We have named these intermediate cells PNH IIIb cells and the more complement-sensitive, common variant, present in ten of 11 examples, PNH IIIa cells.

**DISCUSSION**

One of the facts that has made it difficult to search for the nature of the membrane defect in PNH red cells that renders them susceptible to the lytic action of complement is the existence, in most patients, of a mixture of red cell populations. Thus, the results of biochemical analyses have depended upon the proportion of abnormal cells present, and it has been difficult to define which of the observations were pertinent to abnormal cells. In the past, this difficulty has been minimized by the use of cells of patients with large proportions of abnormal cells. However, such patients are rare, and their rarity makes statistical analysis of findings derived from them difficult in that comparisons among patients cannot be readily made.

Previously, PNH I and PNH II cells have been separated from PNH III cells by differential lysis. PNH III, but not PNH I or PNH II, red cells bind the membrane attack complex when it is initiated in fluid phase by cobra venom factor and are lysed by this process. The PNH III cells in a mixture of cells are lysed by cobra venom factor and serum, and the surviving non–PNH III cells can thus be purified. However, the surviving population(s) (usually PNH I cells) is often the one that is not under investigation. Further, the membranes of the populations that are lysed by complement are contaminated by the presence of complement components; this may interfere with the analysis of their structure.

Suspensions of intact cells enriched by complement-sensitive cells can be obtained by differential centrifugation since the proportion of abnormal cells tends to be greater in those cells. This is because the proportion of abnormal (PNH III) cells that are delivered to the young and hence less dense cells. This is because the number of copies of the antigen is so small compared to the number of copies of the antigen against which the anti-I antibody reacts in this test (probably greater than 600,000 per cell). It is also the low density of acetylcholinesterase molecules that allows normal and PNH I cells to be eluted from the column by increased flow rate alone, without changes in pH or the addition of denaturing agents.

The advantages of such separations in the analysis of the abnormalities of the red cells in PNH are illustrated in the ability to define a new subpopulation of PNH III cells. These cells are so different from the usual PNH III cells that they were not detected in the analysis of complement sensitivity of mixed populations of red cells. Only when the abnormal cells were separated did the difference become apparent. Nevertheless, the identification of these cells has clarified the interpretation of the CLS curves of some patients.

We have also used this technique to separate the abnormal cells for analysis of the presence of decay-accelerating factor (DAF), a membrane protein that normally controls the formation and activity of the C3 convertases of complement. This protein is completely or partially lacking in the complement-sensitive red cells of PNH. By the use of separated populations, we have been able to demonstrate the effects of the absence of DAF and of the depletion of DAF purified from normal cells. The nature of the relationship between acetylcholinesterase deficiency and DAF deficiency is currently under investigation.

By the use of the techniques described herein, purified populations of PNH cells can be obtained from nearly all patients with PNH. This will markedly simplify the analysis of these cells for the nature of their membrane defect(s) and will allow more ready comparison of the cells of different patients.

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