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, 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 radiolabeled 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 (CLS) 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 bound 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 antiserum 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-butyl phthalate, 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 10^7/mL) were incubated with equal volumes of each monoclonal antibody (AE-1 and AE-2) at a dilution that saturated all

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 10^7/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 (P3x63Ag8), the immunoglobulin product of a murine myeloma cell line, was also grown as murine ascites tumors and used as an

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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 I, 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>
</tr>
</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>
</tr>
<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>
</tbody>
</table>

*All antibodies used in saturating amounts, as determined by binding of radiolabeled antimouse Ig.
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 and eluted from the column during the first (slow) phase of the elution process, the other eluted during the rapid elution phase. The cells that were adherent to the column were entirely PNH II cells (Fig 2, curve C). If the cells retained on the column during the first passage are shown in curve D, indicating that the contaminating PNH III cells were not changed by passage through the column. When the red cells lacking acetylcholinesterase were tested 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

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

<table>
<thead>
<tr>
<th>Patient</th>
<th>PNH III in Whole Blood</th>
<th>Percentage</th>
<th>CLS H₅₀ Units</th>
<th>Percentage</th>
<th>CLS H₅₀ Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>V.D.</td>
<td>73</td>
<td>65</td>
<td>36</td>
<td>35</td>
<td>19</td>
</tr>
<tr>
<td>J.P.</td>
<td>58</td>
<td>65</td>
<td>31</td>
<td>35</td>
<td>17</td>
</tr>
<tr>
<td>W.M.</td>
<td>28</td>
<td>49</td>
<td>67</td>
<td>51</td>
<td>18</td>
</tr>
<tr>
<td>J.G.</td>
<td>44</td>
<td>69</td>
<td>38</td>
<td>31</td>
<td>16</td>
</tr>
<tr>
<td>O.P.</td>
<td>42</td>
<td>100</td>
<td>27</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>E.D.</td>
<td>47</td>
<td>100</td>
<td>24</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>J.B.</td>
<td>37</td>
<td>100</td>
<td>37</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>E.G.</td>
<td>61</td>
<td>100</td>
<td>41</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>A.H.</td>
<td>15</td>
<td>100</td>
<td>41</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>M.F.</td>
<td>60</td>
<td>100</td>
<td>27</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>C.K.</td>
<td>&gt;95</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).
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 the young and hence less dense cells. This is because the proportion of abnormal (PNH III) cells that are delivered to the circulation is much greater than the proportion of these cells in the peripheral blood. However, the enrichment for PNH III cells by centrifugation is always incomplete, and the abnormal cells are contaminated by the normal PNH I cells.

The technique described in this paper allows for the first time the separation of the abnormal cells in PNH from the normal PNH I cells by taking advantage of the fact that these abnormal cells lack the protein for the enzyme acetylcholinesterase. Since the antibodies that are used in these studies do not react with the abnormal cells, there is little or no contamination of the membrane with immunoproteins and no disruption of membrane structure. The cells are isolated whole, and the characteristics of lysis in response to complement activation can be analyzed. Further, the presence of the monoclonal antibodies to acetylcholinesterase and antimouse IgG has no effect on the sensitivity of the PNH I and normal red blood cells to complement lysis. This is probably 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 little 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 repletion 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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