Erythrocyte Acetylcholinesterase Deficiency in Paroxysmal Nocturnal Hemoglobinuria (PNH)—A Comparison of the Complement-Sensitive and Insensitive Populations

By T. R. Kunstling and Wendell F. Rosse

Ham and Dingle first demonstrated that the erythrocytes of patients with paroxysmal nocturnal hemoglobinuria (PNH) are unusually susceptible to lysis by complement (C') and antibody. This observation has been confirmed by others. Rosse and Dacie found further that the increased sensitivity to complement lysis depends mainly upon increased efficiency in the lytic activity of complement. They also observed that the blood of patients with PNH contained two populations of erythrocytes; one, the "sensitive" population, is much more susceptible to lysis by complement than normal cells, while the other, the "insensitive" population, is more nearly normal in its susceptibility to complement lysis but is usually not entirely normal. The proportion of cells in each population varies from patient to patient.

The erythrocytes of patients with PNH have also been found to be deficient in the enzyme, acetylcholinesterase (AChE), and the degree of the deficiency has been shown to be related to the severity of the disease. In some patients with PNH, the activity of this enzyme has been found to be within the normal range. These findings were based on studies carried out on red cells from samples of whole blood of PNH patients. Metz et al. also found that after lysis of part of the cells (probably the C'-sensitive cells by acidified serum) the AChE activity of the residual cells was greater than that of the whole blood before lysis.

To define more clearly the relationship of AChE activity to complement lysis sensitivity in each of the populations of PNH red cells, we have separated the "insensitive" and "sensitive" erythrocyte populations and have measured the AChE activity of each population. We have found that the membranes of the complement-sensitive cells contain no measurable AChE activity, and the complement-insensitive cells usually have a slight to moderate decrease in enzyme activity. The degree of enzyme deficiency in these "insensitive" cells is roughly parallel to their moderately increased susceptibility to complement lysis. The total red cell AChE activity of whole blood from PNH patients thus depends both upon the percentage of cells in the complement-insensitive population and upon the AChE activity of these cells.

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**Methods**

**Red Cells**

The cells from seven normal donors and five patients with PNH were examined. The diagnosis of PNH was made in each case on the basis of history, clinical and laboratory examination, and the presence of a positive complement lysis sensitivity test. A brief clinical summary of each patient is given in Table 1. Blood was obtained by venipuncture and stored at 4°C in sterile Alsever’s solution made according to Mayer’s formula. The red cells were prepared for use by washing three times in Veronal buffered saline (VBS) made according to Mayer’s formula.

**Antiserum**

Serum containing a potent cold-agglutinin antibody of high hemolytic titer was obtained from a patient suffering from autoimmune hemolytic anemia, cold-agglutinin antibody type. The antiserum was lyophilized and stored at 0°C. It was diluted to 10 times the original volume with VBS before use. This material was kindly supplied by Prof. J. V. Dacie, Postgraduate Medical School, London.

**Human Complement (C’)**

Blood was taken from normal donors and centrifuged rapidly at 4°C to sediment the erythrocytes. The plasma was then removed and defibrinated by clotting at room temperature. The serum was stored at −80°C and kept at 0°C during the experiments. Once diluted, unused serum was discarded.

**Complement Lysis Sensitivity Test**

The sensitivity of erythrocytes from each patient to complement lysis was determined by a modification of the method of Rosse and Dacie. When PNH cells were examined, four basic dilutions of serum as a source of C’ were used: 1 in 5, 1 in 15, 1 in 50 and 1 in 150. When normal cells were examined only the 1 in 5 dilution was used. For each basic dilution of C’ to be tested, 1.0 ml standard erythrocyte suspension and 1.0 ml cold agglutinin containing antiserum were mixed at 0°C. Immediately thereafter, 0.2 ml portions of this mixture were pipetted, with mixing, into each of six tubes containing respectively, 0.8, 0.7, 0.6, 0.5, 0.3, and 0.1 ml of ice cold VBS in a 0°C bath. In addition, 0.2 ml was pipetted into 1.3 ml VBS (cell control) and 0.2 ml into 1.3 ml. 0.04 per cent NH₄OH (100 per cent lysis control).

Sufficient complement of the dilution being tested was pipetted into each of the six tubes to adjust the final volume to 1.5 ml. After incubation, at 0°C for 12 minutes and at 37°C for 30 minutes, 5.0 ml VBS was added to each tube prior to centrifugation and determination of optical density at a wavelength of 412 μm on a Gilford 300 microsample spectrophotometer. Suitable controls were prepared to permit correction for the color of the C’ solution. Curves relating lysis to C’ concentration were graphed by the method of Rosse and Dacie. The logarithm of y/(1-y), where y is the fraction of cells lysed, is graphed against the logarithm of complement concentration expressed as milliliters of a 1 in 150 dilution of C’ (see Fig. 1). When normal cells were used the resultant graph was a straight line in each case, indicating that a single population of cells was present. When PNH cells were used, complex curves were obtained which indicated the presence of the two populations of cells differing in their sensitivity to C’. Exceptions to these findings will be discussed below.

After the percentage of cells in each population had been determined by this method, the dilution of C’ which would lyse only the "sensitive" population in a 40 per cent suspension of PNH red cells was determined. Twofold falling dilutions of whole human serum from 1 in 1 to 1 in 64 were prepared with VBS; 0.4 ml of diluted serum was incubated with 0.1 ml of a 40 per cent suspension of cells and 0.1 ml of antiserum for 12 minutes at 0°C; then 30 minutes at 37°C. After incubation, the surviving cells were removed by centrifugation and the optical density of the supernatant fluid was determined at
Table 1.—Clinical and Laboratory Data on Patients with P.N.H.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Hemoglobin (Gm. %)</th>
<th>Transfusion Requirement</th>
<th>Duration of Illness</th>
<th>Initial Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.G.</td>
<td>42</td>
<td>M</td>
<td>7.5</td>
<td>Frequent</td>
<td>6 yrs.</td>
<td>Hypoplastic anemia</td>
</tr>
<tr>
<td>H.P.</td>
<td>66</td>
<td>M</td>
<td>8.0</td>
<td>Occasional</td>
<td>9 yrs.</td>
<td>Hemoglobinuria</td>
</tr>
<tr>
<td>G.W.</td>
<td>57</td>
<td>M</td>
<td>8.0</td>
<td>Occasional</td>
<td>4 yrs.</td>
<td>Hemolytic anemia</td>
</tr>
<tr>
<td>P.J.</td>
<td>24</td>
<td>M</td>
<td>9.5</td>
<td>None</td>
<td>3 yrs.</td>
<td>Hemolytic anemia</td>
</tr>
<tr>
<td>C.A.</td>
<td>56</td>
<td>F</td>
<td>12.0</td>
<td>None</td>
<td>1½ yrs.</td>
<td>Hypoplastic anemia</td>
</tr>
</tbody>
</table>

541 mμ using a Gilford spectrophotometer. The dilution of serum found to lyse the percentage of the cells which were in the “sensitive” population was usually found to be 1 in 16. The completeness of the lysis of the “sensitive” cells was determined by performing the C’ lysis sensitivity test on the surviving cells. The results of such an assay are shown in Figure 2.

Complement Lysis of the “Sensitive” Population and Recovery of Ghosts

An erythrocyte suspension, approximately 40 per cent, was made by adding 3.0 ml. VBS to 2.0 ml. packed erythrocytes. From this solution 1.0 ml. was removed for a red blood cell count using a Coulter particle counter Model B; 1.0 ml. was diluted to 1 part in 100 with 0.04 per cent NH₄OH and the optical density determined at wave length 541 mμ on a Gilford spectrophotometer; 1.0 ml. was assayed directly for AChE activity; and 1.0 ml. was subjected to complement lysis as follows: At 0 C. 1.0 ml. of the 40 per cent erythrocyte suspension was mixed with 1.0 ml. of antiserum and 4.0 ml. of a dilution of serum (C’) found to lyse only the sensitive cells. The mixture was incubated at 0 C. for 12 minutes with frequent shaking, then for 30 minutes at 37 C. Following incubation the mixture was spun in a clinical centrifuge for 3 minutes at 1500 rpm. The supernatant fluid was removed, taking care not to remove intact erythrocytes. The remaining unlysed erythrocytes were resuspended in 6.0 ml. VBS and recentrifuged for 3 minutes at 1500 rpm. The supernatant fluid was removed again and combined with the supernatant fluid from the first centrifugation. This suspension, containing the ghosts of lysed cells, was centrifuged 45 minutes at 10,000 rpm, sedimenting the ghosts of the lysed (“sensitive”) cells into a pellet. These ghosts were assayed for AChE activity. A sample of supernatant fluid was diluted 1 in 5 in 0.04 per cent NH₄OH and the optical density was determined at 541 mμ in order to determine the percentage of cells lysed. The unlysed “insensitive” cells were resuspended in VBS to a volume of 10.0 ml. One ml. was removed, diluted 1 in 100 with respect to the original cell concentration in 0.04 per cent NH₄OH for determination of optical density at 541 mμ in order to determine the percentage of cells not lysed by C’. One ml. was taken for a red blood cell count. The remaining unlysed erythrocytes were again recovered by centrifugation and assayed for AChE activity.

To determine whether lysis by C’ alters the AChE activity of normal erythrocytes, 1.0 ml. of 40 per cent suspension of normal red cells was partially lysed by 4.0 ml. of a 1 in 4 dilution of normal serum and a 1.0 ml. of antiserum. The ghosts of the lysed cells were recovered as outlined above and they the unlysed cells were assayed for AChE activity.

AChE Assay

Acetylcholinesterase was assayed using the colorometric method of Pilz.⁸,⁹ Whereas the procedure in Reference 8 was followed exactly, the reagents were prepared according to the methods in Reference 9. There are apparently typographical errors in the directions of the English text of Reference 8. The sensitivity of the assay system was established by testing decreasing numbers of normal cells. AChE activity was detectable in a 1/25 dilution of the original 40 per cent suspension of these cells.
Table 2.—Recovery of Membrane Sialic Acid of PNH Red Cells after Lysis by Complement

<table>
<thead>
<tr>
<th></th>
<th>Per Cent</th>
<th>Sialic acid content</th>
<th>% Sialic acid recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100%</td>
<td>$1.6 \times 10^{-2} \mu M$</td>
<td>100</td>
</tr>
<tr>
<td>Unlysed cells</td>
<td>57%</td>
<td>$0.86 \times 10^{-2} \mu M$</td>
<td>53</td>
</tr>
<tr>
<td>Lysed cells</td>
<td>43%</td>
<td>$0.65 \times 10^{-2} \mu M$</td>
<td>40</td>
</tr>
</tbody>
</table>

Sialic acid Assay

To demonstrate that membranes of erythrocytes lysed by $C^+$ were recovered quantitatively, a 40 per cent erythrocyte suspension was prepared and partially lysed by complement as above, using twice the usual volumes of erythrocytes, antiserum, and serum as a source of complement. Each of the three erythrocyte samples (total cells, lysed “sensitive” cells, and unlysed “insensitive” cells) were then assayed for sialic acid using the thiobarbituric acid method of Warren.10 Prior to assay for sialic acid, the membranes were freed of hemoglobin by washing them twice with 30 ml. of 0.001 M TRIS-0.01 M EDTA buffer at pH 7.4 and the membranes were recovered by centrifugation for 45 minutes at 10,000 rpm. The results are shown in Table 2.

To determine the effect of complete in vitro inhibition of red cell AChE activity upon the sensitivity of the red cells to complement lysis, 2 ml. of a 40 per cent suspension of normal red cells were incubated for 30 minutes at 37 C. with 2 ml. of neostigmine methylsulfate (Prostigmin®) solution containing 0.1 mgm. per ml. in VBS. The samples were divided and the AChE content and the complement lysis sensitivity titers were determined. Control samples were treated in the same manner except that the neostigmine methylsulfate was omitted.

RESULTS

Normal Patients

Complement lysis sensitivity titers of erythrocytes from five normal donors ranged from 0.56 to 1.43 C'LS H50 units (mean 1.05) (Table 3). The AChE activity of the red cells from seven normal donors ranged from 16.0 to 20.1 μequiv. acetylcholine hydrolysed/10⁹ RBC. When normal cells were lysed by complement there was no loss of AChE activity in the membranes of the lysed cells (see Table 4). All the AChE activity of the lysed cells was recovered, confirming that the membranes of cells lysed by $C^+$ are completely recovered by the technics employed.

PNH Patients

Complement lysis sensitivity tests on the cells of three patients yielded biphasic curves. An example is shown in Figure 1. The test on the cells of patient (HP) yielded a triphasic curve, indicating the presence of three populations of cells differing in sensitivity to $C^+$ lysis. The least sensitive population, comprising 20 per cent of his total erythrocytes, had a nearly normal C'LS titer; he had had no transfusions within the previous 4 months to account for the presence of this population. His most sensitive population comprised about 15 per cent of the cells and had a C'LS titer in the range usually found in the sensitive population of other patients with PNH. The remaining 65 per cent of his cells comprised a population with a C'LS titer of
### ERYTHROCYTE ACETYLCHOLINESTERASE DEFICIENCY

**Table 3.—Acetylcholinesterase Activity and Complement Lysis Sensitivity Titers of Normal Donors and of Patients with P.N.H.**

<table>
<thead>
<tr>
<th>Normal Donors</th>
<th>AChE Activity (µ equiv. acetylcholine hydrolysed /10^8 RBC)</th>
<th>C'-L.S. (Hs units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T.K.</td>
<td>19.5</td>
<td>—</td>
</tr>
<tr>
<td>J.P.</td>
<td>17.5</td>
<td>1.35</td>
</tr>
<tr>
<td>B.H.</td>
<td>19.8</td>
<td>—</td>
</tr>
<tr>
<td>J.J.</td>
<td>20.1</td>
<td>0.56</td>
</tr>
<tr>
<td>W.R.</td>
<td>19.2</td>
<td>1.43</td>
</tr>
<tr>
<td>L.G.</td>
<td>16.9</td>
<td>1.14</td>
</tr>
<tr>
<td>W.K.</td>
<td>16.0</td>
<td>0.77</td>
</tr>
<tr>
<td>Mean</td>
<td>18.5</td>
<td>1.05</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>P.N.H. Patients</th>
<th>“Sensitive” Populations</th>
<th>“Insensitive” Populations</th>
<th>C'-L.S. (Hs units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
<td>Whole Blood AChE Activity</td>
<td>Per Cent of Total</td>
<td>Per Cent of AChE units</td>
</tr>
<tr>
<td>A.C.</td>
<td>2.1</td>
<td>60%</td>
<td>0</td>
</tr>
<tr>
<td>H.P.*</td>
<td>5.0</td>
<td>14%</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(2)</td>
</tr>
<tr>
<td>C.W.</td>
<td>14.5</td>
<td>23%</td>
<td>0</td>
</tr>
<tr>
<td>P.J.</td>
<td>7.5</td>
<td>19%</td>
<td>0</td>
</tr>
<tr>
<td>C.A.+</td>
<td>12.2</td>
<td>0%</td>
<td>—</td>
</tr>
</tbody>
</table>

* Patient has two “insensitive” populations (see text).
† Complement “sensitive” population has disappeared (see text).

### Table 4.—Acetylcholinesterase Activity of Normal Erythrocytes Before and After Complement Lysis

<table>
<thead>
<tr>
<th>Control</th>
<th>Per Cent of Cells 100%</th>
<th>Per Cent of recovered AChE Activity 100%</th>
<th>AChE Activity (µ equiv. ACh hydrolysed /10^8 RBC) 22.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unlysed cells</td>
<td>80%</td>
<td>76%</td>
<td>22.0</td>
</tr>
<tr>
<td>Lysed cells</td>
<td>20%</td>
<td>24%</td>
<td>24.0</td>
</tr>
</tbody>
</table>

7.2 C'LS H_50 units, higher than the titer usually seen in “insensitive” cells and lower than that of the “sensitive” cells.

At the time of these investigations, Patient C.A. had a single population of cells which had a moderately increased C'LS titer. She had had pancytopenia in January, 1966 and in April 1966 she was found to have a sensitive population of PNH cells consisting of about 7% per cent of her red cells. During the ensuing year the complement-sensitive population of red cells disappeared.

The complement lysis sensitivity titers of the “sensitive” cells of 4 patients with PNH was found to range from 20–32 units, indicating that these cells are lysed with 1/15th to 1/25th the amount of complement required to lyse normal cells. No measurable AChE activity was found in the ghosts of the “sensitive” cells of any of the patients.

The complement lysis sensitivity titers of the “insensitive” cells of patients...
Fig. 1.—The lysis of normal and PNH red cells by cold-agglutinin antibody and increasing concentrations of human serum as a source of complement. The complement concentration expressed as milliliters of a 1 in 150 dilution of human serum is plotted on the abscissa. A function of lysis, $y/(1-y)$, where $y$ is the fraction of cells lysed, is plotted on the ordinate.

with PNH ranged from 1.5 to 7.2 units and the AChE activity of these cells ranged from 1.1 to 19.3 μequiv. acetylcholine hydrolysed/10^9 RBC (see Table 3). There is in general an inverse relationship between the AChE content and the C'LS titers in the insensitive PNH cells (Fig. 3). In only one patient, the "insensitive" cells appear to be normal both in AChE activity and sensitivity to complement lysis.

Complete inhibition of the red cell AChE activity by neostigmine does not appear to alter the complement lysis sensitivity titer of normal cells (see Table 5).

**DISCUSSION**

These studies show that the red cells of patients with PNH consist of a mosaic of distinct cell populations which differ in their content of acetylcholinesterase as well as in their sensitivity to complement (C') lysis. The cells in the population most sensitive to complement lysis appear to have no AChE activity, whereas the cells in the population relatively insensitive to lysis have a somewhat reduced AChE activity. This is further evidence that the cells in the "insensitive" population are usually not entirely normal as it has been...
Fig. 2.—Complement lysis sensitivity test on PNH cells before and after lysis by antibody and concentrations of serum which lyse only the sensitive population. The data is graphed as in Figure 1. The complement-sensitive population is not present after lysis and the complement lysis sensitivity of the complement-insensitive cells is unchanged.

Fig. 3.—The relationship between the acetylcholinesterase content and the sensitivity to complement of normal and complement-sensitive and complement-insensitive PNH erythrocytes.
Table 5.—Acetylcholinesterase Activity and Complement Lysis Sensitivity of Cell before and after Treatment with Neostigmine Methylsulfate (Prostigmin®)

<table>
<thead>
<tr>
<th></th>
<th>AChE Activity (μequiv. ACh hydrolysed /10⁶ RBC)</th>
<th>C'.L.S. (Ha units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18.8</td>
<td>1.4</td>
</tr>
<tr>
<td>Treated with neostigmine</td>
<td>0.5</td>
<td>1.35</td>
</tr>
</tbody>
</table>

shown previously and confirmed in these studies that these cells are somewhat more sensitive to complement than normal cells.³

The cells of the "insensitive" population of most patients with PNH have some of the characteristics of the red cells of patients with aplastic anemia. Scudamore et al. have shown that red cells from a patient with aplastic anemia may be somewhat deficient in AChE activity.¹¹ These cells appear to be slightly more sensitive to the lytic action of complement than normal cells.³ These similarities are intriguing since it has been repeatedly observed that the PNH can develop in association with aplastic anemia.¹²,¹³

The AChE activity of red cells from whole blood of patients with PNH varies considerably from patient to patient and in general, the more severe the disease, the lower the AChE activity.⁵,⁶ This can be explained in part by the fact that the clinical manifestations of the disease are, in general, related to the percentage of complement-sensitive cells.³ Since these cells have no detectable AChE activity, the greater their proportion in the blood, the lower the AChE activity of the red cells from whole blood. The magnitude of complement-sensitive population determines to a major degree both the clinical severity of the disease and the diminution in AChE activity of the cells from whole blood.

An etiologic relationship between diminished AChE activity and increased sensitivity to complement lysis by no means is established by these experiments. Two possibilities exist: 1) the deficiency of AChE in the membrane of the PNH red cell is directly or indirectly responsible for the increased susceptibility to lysis by C' and hence to their decreased survival in vivo, or 2) the membrane defect which increases the susceptibility of PNH red cells to lysis by C' also makes them deficient in AChE activity but there is no causal relationship between the lack of AChE activity and the increased susceptibility to lysis by complement.

Chemical reduction of the erythrocyte AChE activity to 30-50 per cent of normal by the experimental or accidental administration of organic phosphates (parathione or octamethylpyrophosphoramide (OMPA), does not appear to reduce the survival of normal red cells nor to increase their sensitivity to complement lysis as judged by the fact that the acidified serum lysis test remains negative.¹⁴,¹⁰ In addition several members of a family have been found to have a partial deficiency in erythrocyte AChE activity (20-40 per cent of normal) but none showed evidence of increased hemolysis or lysis of red cells in acidified serum.¹⁶ It is possible that the degree of inhibition deficiency seen was insufficient to decrease survival since the present studies show that the red cells in PNH which have a markedly reduced survival in
vivo have no detectable enzyme activity in the membrane. On the other hand, virtually complete inhibition of AChE activity of normal cells in vitro by neostigmine methylsulfate (Prostigmin®) does not alter the C’ lysis sensitivity of these cells.

These findings, while not conclusive, suggest that inhibition of the enzyme activity either stereochemically or genetically probably does not, per se, lead to sensitivity to complement lysis in vitro or hemolysis in vivo.

**SUMMARY**

1. The complement-sensitive and -insensitive cells of patients with paroxysmal nocturnal hemoglobinuria have been separated by differential lysis using a cold agglutinin antibody and limiting concentrations of complement. The erythrocyte acetylcholinesterase (AChE) activity of each population of cells was determined.

2. The membranes of the complement-sensitive cells characteristic of PNH completely lack AChE activity.

3. The membranes of the complement-insensitive cells usually contain less AChE than normal and the degree of decrease roughly parallels the degree of increased sensitivity to complement.

4. Lysis of normal cells by complement does not alter the AChE activity of their membranes.

5. The inhibition of AChE activity by neostigmine methylsulfate (Prostigmin®) does not increase the sensitivity of normal red cells to lysis by C’.

**SUMMARIO IN INTERLINGUA**

1. Le cellulas complemento-sensibile e complemento-insensibile de patientes con paroxysmic hemoglobinuria nocturne esseva separate per lyse differential, con le uso de un anticorpore anti cryoaglutinina e concentrationes limitatori de complemento. Le activitate de acetylcholinesterase erythrocytic (AChE) de cata-un del populationes cellular esseva determinate.

2. Le membranas del celular complemento-sensibile que es characteristic de paroxysmic hemoglobinuria nocturne es completemente dispoviste de activitate de AChE.

3. Le membranas del cellulari complemento-insensibile contine usualmente minus AChE que es normal, e le grado de declino es grossiermente parallel al grado de augmento in le sensibilitate pro complemento.

4. Le lyse de cellulas normal per complemento non altera le activitate de AChE de lor membranas.

5. Le inhibition de activitate de AChE per medio de methylsulfato de neostigmina (Prostigmina®) non aumenta le sensibilitate de normal erythrocytos pro le effecto lytic de C’.

**REFERENCES**


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