The Acetylcholinesterase Defect in Paroxysmal Nocturnal Hemoglobinuria: Evidence That the Enzyme Is Absent From the Cell Membrane

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Paroxysmal nocturnal hemoglobinuria (PNH) is a myelodysplastic disease characterized by erythrocytes that show abnormally increased sensitivity to complement-mediated lysis. Complement-sensitive PNH erythrocyte membranes have previously been shown to lack acetylcholinesterase (AchE) activity, but the molecular basis of this deficiency has been unclear. We have used monoclonal antibodies to four different epitopes on the AchE molecule to show that abnormal PNH erythrocytes failed to bind these antibodies.

A C T Y L C H O L I N E S T E R A S E (AcChE) EC 3.1.1.7) is found on the plasma membrane surface of all mammalian erythrocytes. However, the role of erythrocyte AchE remains unknown. Deficiency of erythrocyte AchE activity has been shown to occur as both inherited and acquired phenomena. Although many diseases have been described as associated with decreased AchE activity, paroxysmal nocturnal hemoglobinuria (PNH) is the only disease in which decreased AchE activity has been consistently found.

PNH is characterized by the existence of at least two populations of erythrocytes (E)—one of normal E (PNH I cells) and the other(s) of cells that exhibit varying degrees of abnormal sensitivity to lysis by complement (PNH II and III cells). Auditore and Hartmann first described decreased AchE activity in the blood and bone marrow of patients with PNH in 1959. The decrease in enzyme activity was soon shown to be proportional to the severity of the disease. Kunstling and Rosse showed that the abnormal complement-sensitive E of PNH patients had no detectable AchE activity, while AchE activity was present on the normal complement-insensitive (PNH I) E. However, the cause and possible importance of the loss of AchE activity on PNH cells have remained unclear. Investigators have postulated that the enzyme might be structurally abnormal, that its active sites might be blocked by an unknown factor, or that it is abnormally oriented in the lipid bilayer.

In the present study, we have used four monoclonal antibodies to four different epitopes on human E AchE to show that abnormal PNH E appear to lack the AchE protein rather than to have a protein with abnormal or no enzyme activity.

MATERIALS AND METHODS

Acquisition and use of monoclonal antibodies. All anti-AchE antibodies and their patterns of reactivity have been previously described by Fambrough and colleagues. The AE-1 and AE-2 cell lines were acquired from the American Type Culture Collection (Rockville, Md) as cells in culture media and grown as ascites tumors in BALB/c mice to produce high-titered antisera as ascites fluid. P3 x 63Ag8(11) ascites was used as an isotype-specific control in all experiments. In addition, purified AE-1, AE-2, AE-3, and AE-4 IgG (obtained by diethylaminoethyl [DEAE] chromatography from ascites) were the generous gift of Dr D. Fambrough (Carnegie Institute of Washington, Baltimore).

Assays to measure cell surface reactivity with anti-AchE antibodies. Cell surface antibody reactivity was measured by radioimmunoassay as previously described. Briefly, 5% (vol/vol) suspensions of washed E were incubated for 30 minutes at room temperature with dilutions of fluid in triplicate wells of V-bottom microtiter plates (Dynatech, Alexandria, Va). E were washed three times in Dulbecco’s phosphate-buffered saline (DPBS) with 1% bovine serum albumin (BSA) and 0.1% gelatin. E were then resuspended in DPBS, and 125I-labeled affinity-purified F(ab’2) anti-mouse Ig antibodies (Amersham Corporation, Arlington Heights, Ill) were used to detect binding of antibody. Specific binding was determined as positive Δ cpm, where Δ cpm = mean cpm experimental – mean cpm control (P1).

Alternatively, radioimmunoassays were done in tubes by the same method, except that after incubation with 125I-labeled anti-mouse Ig, triplicate aliquots of cells were spun through a 1.5:1.0 mixture of N-butyl and bis 2-ethylhexyl phthalate oils in plastic microfuge tubes, and the tips containing the pellets were then cut and the radioactivity measured. In some experiments, aliquots of E were pretreated with 2-aminoethylisothiouronium bromide (AET) as previously described prior to being used in antibody-binding assays.

Radioiodination of erythrocytes. Erythrocytes were obtained from ethylene diamine tetra-acetic acid (EDTA)-anticoagulated or acid-citrate-dextrose–preserved whole blood by separation through PBS 5 mmol/L of EDTA, 1% dextrose (T500, Pharmacia, Uppsala, Sweden) for 30 to 60 minutes at 4°C. After removal of the leukocyte compartment and the top 10% of the erythrocyte compartment, E were suspended and washed three times in PBS. Approximately 10% of the E column was aspirated with the supernatant fluid to ensure that the top 10% of the erythrocyte compartment, E were suspended and washed three times in PBS. Approximately 10% of the E column was aspirated with the supernatant fluid to ensure removal of leukocytes and platelets. E were then suspended in PBS and adjusted to 107 cells per milliliter by counting on an ELT8 counter (Ortho Diagnostics, Raritan, NJ). E were then iodinated with lodo-Gen (Pierce Chemical Co, Rockford, Ill) as described by Markwell and Fox. Briefly, E were radiolabeled using 0.5 to 1.0 mCi 125I per 1.0 to 1.5 x 108 cells in a volume of 1.0 to 1.5 mL. All radioiodinations were performed for ten minutes at 25°C and followed by two 15-mL washes in PBS plus 5 mmol/L of NaI and a final wash in PBS.

Radioimmuno-precipitation of E AchE. Samples containing 5 x 108 125I-labeled E were solubilized in 400 μL of 150 mmol/L of NaCl, 50 mmol/L of Tris, pH 8.0, 1% deoxycholate, 3 mmol/L of phenylmethyl-sulfonyl fluoride for 30 minutes at 4°C on a rocker platform. After 20 minutes’ centrifugation at 27,000 g, super-

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nates were used for immunoprecipitation experiments. Proteins that bound staphyloccocal protein A nonspecifically were removed by incubation of each aliquot of lysate with 100 μL of 10% suspension of washed Staphylococcus aureus Cowan strain 1 (SAC) Calbiochem-Behring, La Jolla, Calif) for one hour at 4 °C, after which the SAC was pelleted by centrifugation. A saturating amount of anti-AchE or P3 (control) antibody was then added to each supernate. After one hour, 100 μL of 10% SAC previously coated with goat anti-mouse IgG plus IgM (Tago, Burlingame, Calif) was added to each tube and incubated overnight at 4 °C. The SAC was then pelleted, resuspended, and washed three times in 150 mmol/L of NaCl, 50 mmol/L of Tris, 2% sodium dodecyl sulfate (SDS), 15% glycerol, pH 6.8, with or without 5% 2-mercaptoethanol. Eluates were analyzed by electrophoresis through discontinuous SDS-polyacrylamide slab gels (PAGE) consisting of a 5% to 12% linear gradient over laid with a 3% stacking gel. At least one lane per gel contained a [14C]methylated protein mixture (Amersham) for molecular weight standards. After drying, gels were autoradiographed as required to visualize immunoprecipitated protein bands and radiolabeled molecular weight standards.

Separation of erythrocytes by Percoll density centrifugation. Thirty-five milliliters of Percoll (density, 1.130 g/mL; Pharmacia) was diluted with 20 mL of diatrizoate meglumine and sodium (600 mg/mL), mixed with 1 g of BSA, 0.5 g of glucose, and brought to 100 mL with H2O. Ten milliliters of this solution were placed in a 15-mL tube and mixed with 150 μL of packed E. This was then centrifuged at 34,500 g for 15 minutes at 4 °C. The topmost 1 mL, enriched for reticulocytes, was discarded. The remaining Percoll contained an upper portion of approximately 2.5 mL containing several visible E bands; these upper bands were collected and pooled to comprise the light E fraction. Below this was approximately 2 mL with no visible E bands; this was also discarded. The remaining bands in the lower portion were then pooled to comprise the dense E fraction. The erythrocytes in these two fractions were then washed in PBS 3 times. Each was then solubilized in NaCl/Tris/1% deoxycholate (DOC) buffer as above and used for immunoprecipitation experiments.

Separation of PNH E by complement lysis. For immunoprecipitation experiments, 3 mL of PNH E containing approximately 50% complement-sensitive E and 2 mL of normal donor E were radiolabeled with 125I as above. One milliliter of each was used for immunoprecipitation with one anti-AchE and one control antibody. The 2 mL of PNH E remaining were washed and resuspended to a final volume of 1 mL in veronal-buffered saline, 0.1% gelatin (GVB)-EDTA. Complexes (CoFBb) ofcobra venom factor (Cof) and activated factor B (Bb) were made by adding 150 μL of CoF Naja naja (1.02 mg/mL) to 500 μL of type-specific serum and incubating for 30 minutes at 37 °C (C. Parker, W.F. Rosse, P.J. Sims, and T. Weidner, manuscript in preparation). Another 2 mL of serum chelated by adding 200 μL of 0.2 mol/L of EDTA to 2 mL of serum and 650 μL of CoFBb complexes were added to the PNH E suspended in GVB-EDTA, which were then mixed by vortexing and incubated for 30 minutes at 37 °C with frequent agitation. After completion of the incubation period, the cell suspension was layered over 2 mL Hypaque-Ficoll and centrifuged at 2,000 rpm for 20 minutes to separate lysed from unlysed cells. Cell membranes from the Hypaque-Ficoll/surface interface and pelleted whole E were then washed in PBS 3 times. Each was then solubilized in NaCl/Tris/1% deoxycholate (DOC) buffer as above and used for immunoprecipitation experiments.

Normal donor E were incubated with 1 mL of a 1/25 dilution of purified cold agglutinin in 1 mL of 1:1 dilution of type-specific serum in order to obtain approximately 50% lysis. Lysed and unlysed cells were then separated, washed, and used for immunoprecipitation experiments as above.

E AchE enzyme assay. Erythrocytes were assayed for AchE activity by a modification of the method of Eillman et al.17 Washed E were suspended in PBS so that the cell suspension had an optical density (OD) of 1.7 (1.67 x 106 per milliliter). One-tenth-milliliter cell suspension was then added to 2.9 mL of H2O. One-tenth milliliter of lysed cell suspension was then added to one tube containing 2.3 mL of 0.08% dithiobisnitrobenzoate (DTNB) and to a control tube containing 2.3 mL of 0.08% DTNB and 2.5 mL absolute alcohol. One-tenth milliliter of 0.02 mol/L acetylthiocholine iodide was then added to each tube and incubated at 37 °C for exactly 15 minutes, after which 2.5 mL of absolute alcohol was added to the test sample to stop AchE activity. The ODs of both samples were then obtained at 412 nm. Membranes of lysed cells were washed thrice to remove hemoglobin and were resuspended to volume. Each suspension was then assayed for protein by Bio-Rad protein assay. AchE activity was calculated as mmol/60 min/mg protein by the following equation:

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\text{AchE activity} = (\text{OD detected} - \text{OD blanks}) \times 458 \text{ mmol/60 min/mg}.
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RESULTS

Initial radioimmunoassay binding studies using AE-1 and AE-2 antibodies indicated that PNH E (from six and seven patients, respectively) bound less antibody than did normal E, and that the decrease in antibody binding was roughly proportional \((r = .67 \text{ for AE-1}, r = .79 \text{ for AE-2})\) to the percentage of abnormal complement-sensitive cells circulating.

![Fig 1. Reactivity of PNH erythrocytes with anti-AchE antibodies AE-1 (A) and AE-2 (B).](image-url)
ing (as determined by the complement lysis sensitivity assay) (Fig 1). These results suggested that AchE antigen activity as well as enzyme activity was decreased on PNH E. Binding of AE-1 and AE-2 antibodies to normal E was also markedly decreased—to 22.6% and 20.5% of original activity, respectively—by treatment of E with AET, which has been previously shown to destroy AchE enzyme activity.

AE-1 and AE-2 antibodies were then used to immunoprecipitate AchE from normal radiolabeled E; both antibodies precipitated an approximately 74-kilodalton (kd) protein when analyzed under reducing conditions (Fig 2A). Under nonreducing conditions, AchE from normal E appeared to have a molecular weight of approximately 155,000 daltons (Figure 2B). When E from PNH patients with >90% complement-sensitive cells (type II or III) were used in immunoprecipitation studies, either no AchE or barely detectable amounts of AchE were precipitated by AE-1 and AE-2 antibodies (Fig 3). E from patients with at least 30% normal red cells had reduced but detectable AchE as assayed by immunoprecipitation with AE-1 and AE-2 antibodies (Fig 3).

Two techniques were then used to separate normal from complement-sensitive PNH E. First, we separated washed PNH E by Percoll density centrifugation. Initial work with normal E separated into fractions by Percoll density centrifugation had shown that the topmost reticulocyte-enriched band often had increased AchE activity, while most of the light fractions of the PNH E had reduced AchE activity (F. L. Chow and M. Morrison, unpublished observations, 1983). Therefore, the topmost (reticulocyte-enriched) fraction of PNH E was discarded to avoid increased AchE due to the presence of reticulocytes. After discarding the top fraction, the remaining light upper and dense lower fractions were assayed for percentage of complement-sensitive cells and for AchE antigen by immunoprecipitation. We found that in a typical experiment, E from a normal donor showed equal amounts of precipitable AchE in the upper and lower Percoll fractions (data not shown). However, E from patients with PNH showed no precipitable E in the upper fraction.
and nearly normal AchE in the lower fraction (data not shown). However, Percoll density separation, although it produced an upper fraction enriched for complement-sensitive cells without producing any detectable cell membrane alterations, did not entirely separate the normal from abnormal cells.

Essentially complete separation of PNH I and III cells was achieved by actual lysis of abnormal complement-sensitive cells with cobra venom-activated complement. Immunoprecipitable AchE of lysed and unlysed E was then compared with that of normal E before and after lysis with antibody and complement. Results of one such experiment, using AE-2 antibody, are presented in Fig 4. When E from a normal donor were used as a source of immunoprecipitable AchE, washed E, E lysed by antibody and complement, and E exposed to but not lysed by CoFBb showed strong bands of AchE. However, washed E from a PNH patient, with 70% abnormal complement-sensitive cells, gave a much weaker AchE band (whole E). After lysis of the abnormal cells by cobra venom-activated complement, the unlysed (normal PNH I) cells gave a strong AchE band comparable to that obtained using an equal number of normal donor E. On the other hand, the PNH cells lysed by complement showed no immunoprecipitable AchE. When cells from the same patient were tested for AchE enzyme activity before and after complement lysis, the following results were obtained. Unseparated (whole) E with 70% abnormal cells had 0.223 mmol/60 min/mg or 22% of the mean normal AchE activity of 0.975 mmol/60 min/mg ± 0.063 (SEM) (Table 1). After complement lysis of abnormal cells and separation of unlysed cells from lysed cells, the unlysed cells had normal AchE activity, while the lysed cells had barely detectable AchE activity (Table 1). Thus, immunoprecipitation and enzyme assay data suggest that the lack of enzyme activity is due to the lack of the AchE protein on the cell membrane surface. Similar experiments using PNH E separated by complement lysis or E from patients with >95% abnormal E (type II or III) further confirmed that no immunoprecipitable AchE was detectable on type II or III PNH E and that immunoprecipitable AchE was absent uniformly when AchE enzyme activity was absent. Further studies using purified AE-3 and AE-4 also showed that AE-3 and AE-4 antibodies—which identify two additional epitopes on the AchE molecule—also showed reduced binding to E from PNH patients and failed to immunoprecipitate AchE from deoxycholate lysates of complement-sensitive PNH E lacking AchE enzyme activity, although these antibodies did precipitate AchE from normal E (data not shown).

**DISCUSSION**

The four anti-AchE antibodies used in this study have been previously shown to be against four different epitopes on AchE by both blocking studies and phlyogenic data. Our immunoprecipitation data further showed that these antibodies all precipitated bands of identical molecular weight from normal red cells in agreement with that previously described for AchE. All four antibodies precipitated only dimeric AchE under the conditions described above; however, analysis of precipitates under reducing conditions showed uniformly complete separation of AchE dimers into monomers. The addition of larger amounts of radioactive or nonradioactive iodine during radiolabeling or washing of E caused immunoprecipitation of some monomeric AchE (lane G). while complement-sensitive (type III) PNH cells contained no immunoprecipitable AchE. A representative radiolabeling pattern of normal cells is shown in lane J.

Table 1. Acetylcholinesterase Activity in PNH Erythrocyte Membranes Before and After Lysis by Cobra Venom Factor-Activated Complement

<table>
<thead>
<tr>
<th></th>
<th>Enzyme Activity (mmol/60 min/mg)</th>
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<tbody>
<tr>
<td>Normal donors (n = 5)</td>
<td>0.975 ± 0.063 (SEM)</td>
</tr>
<tr>
<td>Unseparated PNH E (70% type III)</td>
<td>0.223</td>
</tr>
<tr>
<td>Unlysed PNH E (type I)</td>
<td>0.956</td>
</tr>
<tr>
<td>Lysed PNH E (type III)</td>
<td>0.011</td>
</tr>
</tbody>
</table>

This table presents data on one patient’s erythrocyte membranes. Immunoprecipitation analysis of this patient’s erythrocyte membranes is shown in Fig 4.
teins. The defects thus far described include decreased AchE activity, qualitative abnormalities of glycoporin, and reduced activity or absence of two complement regulatory factors—the C3b receptor (CR1) and the decay-accelerating factor (DAF). However, the cause of these multiple membrane protein abnormalities remains obscure. The present study suggests that, in the case of AchE, the protein is absent from the cell membrane surface. However, other possibilities remain. First, AchE has been shown to have a very small hydrophobic intramembranous domain. However, none of the antibodies used in this study was directed toward this domain. Thus, it is possible that the hydrophilic domain—containing the active enzymatic site as well as the epitopes identified by the AE antibodies—is absent from complement-sensitive PNH cells, while the hydrophobic segment of the molecule remains intact. This could occur if the AchE molecule were abnormally sensitive to partial degradation, due to abnormalities of the AchE molecule or due to changes of neighboring molecules in the cell membrane. Second, the protein may be situated in the membrane abnormally, so that it is inactive as well as unavailable to both antibody interaction and iodination. However, AchE activity is absent from complement-sensitive PNH E, which are lysed with or without solubilization of membrane proteins by detergent. Thus, it is unlikely that the AchE abnormality is dependent on abnormal position of the protein in the membrane or on presence of a blocking factor on the cell surface. The AchE protein—as well as others—may fail to be inserted into the E membrane, perhaps due to abnormal glycosylation events during protein processing. It has been shown that, when glycosylation of membrane proteins in tissue culture cells is blocked by tunicamycin, the proteins fail to be inserted into the cell membrane. Incomplete glycosylation may also affect the stability of glycoproteins, leading to rapid degradation. Although glycoporins A, B, and C appear to be present in normal amounts on PNH cells as assayed by binding of monoclonal anti-glycoporin antibodies (M.J. Telen and W.F. Rosse, unpublished observations, 1984), it may be that a crucial glycosylation step in the processing of AchE protein is not carried out in PNH cells, leading either to failure to insert the protein into the membrane or to instability of the protein after insertion. A similar mechanism may also be responsible for the deficiency of DAF. The fact that protein and lipid glycosylation abnormalities in tumor cells have been extensively described also supports the hypothesis that such abnormalities may be crucial in PNH. Although PNH erythrocytes are not malignant per se, PNH may occur as a premalignant syndrome. Examples of glycosylation abnormalities in malignant hema-

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