Structural and Functional Differences Between Decay-Accelerating Factor and Red Cell Acetylcholinesterase

By Jeremy Sugarman, Dana V. Devine, and Wendell F. Rosse

The abnormal erythrocytes in paroxysmal nocturnal hemoglobinuria (PNH) are devoid of decay-accelerating factor (DAF), which modulates both the classical and alternative pathways of the complement cascade. DAF prevents the amplification of the C3 convertase at C3 by interaction with either C4b2a, the classical pathway C3 convertase, or C3bBb, the alternative pathway C3 convertase. The insertion of DAF into the membranes of PNH erythrocytes can partially ameliorate the sensitivity to lysis by complement. Since the DAF defect in PNH parallels the AChE defect, it has been suggested that the two activities reside on the same protein. We performed both functional and immunologic studies to explore the relationship between these two proteins and have demonstrated that the two are different proteins.

MATERIALS AND METHODS

Cells and reagents. For studies using human RBC, blood was collected fresh each day from normal healthy volunteers and anticoagulated in EDTA. Sheep blood was obtained locally and stored in acid citrate dextrose.

Monoclonal antibodies against AChE, AE1, and AE2 were obtained from the American Type Culture Collection (Rockville, Md). A polyclonal antibody to DAF was prepared in rabbits as described in Nicholson-Weller et al. Antiglycophorin activity was removed from the antiserum by absorption against Type III PNH cells which were purified by the method of Chow et al. Normal rabbit serum (NRS) and a mouse monoclonal antibody (P3) with no reactivity on RBC served as controls. Staphylococcal protein A (SPA) was radiolabeled with 125I using iodogen. Guinea pig C1 and C2 were prepared as previously described.

Effect of anti-DAF or anti-AChE antibody binding on AChE activity. RBC were washed three times in 0.01 mol/L phosphate buffer, pH 8.0, (PB) and resuspended to 1.5 x 10^9/mL. Antibody binding was performed by incubating one part anti-DAF (1:100), AE-2 (1:400), or nonimmune antibody diluted in phosphate-buffered saline containing 1% bovine serum albumin (PBS-BSA) with two parts washed RBC for 30 minutes at RT. Cells were then washed twice in PBS and diluted 1:600 in PB.

The functional activity of red cell AChE was determined using the method described by Ellman. Briefly, 10 μL of 0.1% quinidine sulfate in absolute ethanol, 25 μL of 0.01 mol/L dithiobisnitrobenzoate (Sigma Chemicals, St. Louis, Mo), and 3 mL of the cell suspension were placed in a cuvette and the reaction was initiated by the addition of 20 μL acetylthiocholine (Sigma). The reaction was monitored at 412 nm for 6 minutes using an LKB Ultrospec which was linked to an Apple II computer with a reaction rate measurement program (Biochrom, Cambridge, England, package 4073-260). The number of moles of substrate hydrolyzed per minute per red blood cell (AU) was determined for each sample according to the equation derived by E10m.

Competitive binding of anti-DAF and AE2. Washed RBC at 5 x 10^9/mL were incubated with an equal volume of anti-DAF (1:100) or PBS-BSA for 30 minutes at RT, washed three times in PBS, then resuspended to the starting volume. Each sample was then mixed with an equal volume of AE2 (1:100, 1:200, or 1:400) or PBS-BSA and incubated and washed as described above. To determine binding specificity of the antibodies described above, cells were incubated with NRS or P3 during the first incubation and PBS-BSA in the second. One hundred μL of each sample was then incubated with 100 μL of either 125I-labeled F(ab')2, sheep antimmune immunoglobulin (Amersham, Arlington Heights, Ill) or 125I-labeled SPA for 30 minutes at RT. Three aliquots of 30 μL each of sample were spun through phthalate oils [1.5 parts n-buty phthalate : 1.0 part bis(2-ethylhexyl) phthalate] to separate bound from unbound radiolabeled probe and the cell pellets counted in a gamma counter. The percent of inhibition of binding was determined by dividing the cpm of the samples incubated with anti-DAF then AE2 by the cpm of the control incubation (NRS then AE2). The experiment was repeated.
reversing the order of antibody incubations. Here, each sample was first incubated with AE2 (1:100), and then with anti-DAF (1:200, 1:500, or 1:1000).

Effect of anti-DAF or anti-AChE antibody on complement lysis sensitivity. A modified version of the complement lysis sensitivity (CLS) test\(^1\) was performed using a total reaction volume of 375 \(\mu\)L, but leaving the proportion of all materials used the same. To determine the effect of DAF or AChE inhibition on the CLS test, cells assayed in the CLS were treated with anti-DAF (1:100), a mixture of AE1 and AE2 (each at 1:100), or nonimmune IgG as described in the competitive binding studies.

Effect of AChE on the decay of C4b2a. Sheep erythrocytes bearing classical pathway C3 convertases (C4b2a) were prepared by reacting sheep RBC suspended in veronal-buffered saline (2.5 mmol/L sodium barbital, 75 mmol/L NaCl, pH 7.5, 0.05% w/v gelatin, 2.5% w/v dextrose, 0.5 mmol/L MgCl\(_2\), 0.15 mmol/L CaCl\(_2\)) (DGVB) with rabbit antisympathetic antibody prepared in this laboratory, guinea pig C1, and human C4 (Cordis Laboratories, Hialeah, Fla). Guinea pig C2 was added to form sufficient C4b2a to produce approximately 1.0 hemolytic site per cell when the convertase sites were developed with EDTA-chelated guinea pig serum diluted 1:12 in EDTA-veronal buffered saline (5 mmol/L sodium barbital, 0.15 mol/L NaCl, pH 7.5, 0.1% (w/v) gelatin, 0.04 mol/L EDTA) (EDTA-GPS).

The functional activity of C4b2a was measured using the method of Nicholson-Weller et al\(^8\) for the detection of DAF activity. Red cell AChE (Sigma) was solubilized in DGVB containing 0.01% NP-40. This AChE preparation contained at least 15% AChE as determined by densiometric scan of Coomassie blue staining of the material after SDS-PAGE. EAC142 were incubated with an equal volume of AChE suspension or buffer containing only 0.01% NP-40. Aliquots were removed at various times up to 30 minutes and the residual C4b2a sites developed with EDTA-GPS.

Sequential radioimmunoprecipitation. RBC were radioiodinated as previously described\(^9\) using the iodogen (Pierce Chemicals; Rockford, Ill) method. Samples containing 1 \(\times\) 10\(^4\) \(^{125}\)I-labeled RBC were solubilized in RIP buffer (0.15 mol/L NaCl, 50 mmol/L Tris, 5 mmol/L EDTA, 0.5% NP-40, 0.1% w/v gelatin, pH 7.4) for 30 minutes at RT, and then centrifuged at 4 °C at 15,000 \(\times\) g for 20 minutes. The supernate was incubated with formalin-fixed Staphylococcus aureus strain Cowan I (SAC) (Calbiochem-Behring; La Jolla, Calif) to reduce nonspecific protein binding. After removing the SAC, the supernate was incubated with either 10 \(\mu\)L of anti-DAF or NRS and SAC. Antibody and SAC incubation was repeated four times. Following the last incubation, the supernates were divided in half and incubated with either 20 \(\mu\)L AE2 or P3 or 10 \(\mu\)L anti-DAF or NRS and SAC previously coated with goat antismouse IgG and IgM (Tago; Burlingame, Calif). After washing in SAC buffer all the SAC pellets were boiled in SDS sample buffer to release antigen-antibody complexes. The supernatant fluid was then analyzed by SDS-PAGE (7.5% resolving gel) under nonreducing conditions. The gels were dried and autoradiographed.

Table 1. The Effect of Antibodies to AChE or DAF on the Activity of AChE

<table>
<thead>
<tr>
<th>Normal RBC Pretreated With</th>
<th>AChE Activity (moles hydrolyzed/min/cell (\times) 10(^{-14}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-AChE (AE-2)</td>
<td>1.60</td>
</tr>
<tr>
<td>P3 control*</td>
<td>2.21</td>
</tr>
<tr>
<td>Rabbit anti-DAF</td>
<td>2.65</td>
</tr>
<tr>
<td>Normal rabbit serum</td>
<td>2.08</td>
</tr>
</tbody>
</table>

*Monoclonal antibody lacking reactivity with RBC.

In a similar fashion, a sequential RIP of AChE followed by DAF was performed. Here, the initial precipitation consisted of 20 \(\mu\)L of AE2 or P3 and antismouse-coated SAC. The antibody and antismouse-coated SAC incubations were repeated twice. The resulting supernates were divided in half and incubated with 10 \(\mu\)L of either anti-DAF or normal rabbit serum and SAC. Pellets were prepared for SDS-PAGE and electrophoresis and autoradiography was performed as described above.

RESULTS

The AChE activity of untreated normal RBC was 2.87 \(\times\) 10\(^{-13}\) moles of substrate hydrolyzed per minute per cell (AU). AChE-deficient PNH Type III cells had an activity of 0.18 AU. Incubation of normal RBC with AE-2 decreased the AChE activity from 2.21 AU to 1.60 AU, while incubation with anti-DAF did not inhibit AChE activity (Table 1).

The binding of anti-DAF to RBC did not inhibit subsequent binding of AE2 at any of the antibody concentrations tested (Table 2). Likewise, binding of AE2 did not inhibit subsequent binding of anti-DAF.

The effect of anti-DAF, AE-1, and AE-2 on the CLS test is shown in Fig 1. Incubation of RBC with two monoclonal antibodies to AChE did not change their sensitivity to complement lysis. In contrast, inhibition of DAF by anti-DAF caused an increased susceptibility to complement lysis.

The rate of decay of the classical pathway convertase from the surface of sheep RBC was not affected by the addition of human red cell AChE to the reaction mixture (Fig 2). This was true at all concentrations of AChE tested up to 10 mg/mL. The decay rates in the presence of 0.01% NP-40 were similar to those measured in the absence of the detergent, however, the overall amount of lysis was slightly greater. This assay readily detected DAF solubilized from the membranes of normal RBC.

As can be seen in Fig 3, all DAF is precipitated from the cell lysate after three incubations in anti-DAF. However, AE2 is still able to precipitate AChE from DAF-free lysate.

Table 2. Competitive Binding of Anti-DAF and Anti-AChE to Normal Erythrocytes

<table>
<thead>
<tr>
<th>First Incubation</th>
<th>Second Incubation</th>
<th>Third Incubation</th>
<th>% Inhibition of Binding of Second Incubation Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-DAF*</td>
<td>anti-AChE (1:1000)</td>
<td>125I-antimouse IgG</td>
<td>4%</td>
</tr>
<tr>
<td>Anti-DAF</td>
<td>anti-AChE (1:200)</td>
<td>125I-antimouse IgG</td>
<td>5%</td>
</tr>
<tr>
<td>Anti-AChE</td>
<td>anti-AChE (1:400)</td>
<td>125I-antimouse IgG</td>
<td>1%</td>
</tr>
<tr>
<td>Anti-DAF</td>
<td>anti-AChE (1:200)</td>
<td>125I-SPA</td>
<td>0%</td>
</tr>
<tr>
<td>Anti-AChE</td>
<td>anti-AChE (1:500)</td>
<td>125I-SPA</td>
<td>6%</td>
</tr>
<tr>
<td>Anti-DAF</td>
<td>anti-AChE (1:1000)</td>
<td>125I-SPA</td>
<td>6%</td>
</tr>
</tbody>
</table>

*Anti-DAF was used in the first incubation at 1:100.
†Anti-AChE (AE-2) was used in the first incubation at 1:100.
The effect of antibodies to DAF or AChE on the sensitivity of normal erythrocytes to lysis by complement. Cells were pretreated with anti-DAF, monoclonal antibodies to AChE (AE-1 + AE-2), or P3, a monoclonal antibody that does not react with RBC. The cells were then exposed to cold agglutinin anti-I antibody and dilutions of normal human serum as a source of complement. The fraction of cells lysed was determined by spectrophotometric measurement of hemoglobin release and the data analyzed as in Rosse and Dacie. The curve shown for cells treated with P3 is the same as that for cells exposed only to buffer.

Similarly, when all AChE was removed from the lysate by precipitation with AE2 (Fig 4), DAF was precipitated from that cell lysate. Polyclonal anti-DAF precipitated a band with a relative mobility of 63,000 daltons while AE2 precipitated AChE in its dimeric form at 140,000 daltons. Anti-DAF did not precipitate a similar band from PNH erythrocytes (data not shown).

The effect of AChE on the rate of decay of the classical pathway C3 convertase. Sheep erythrocytes bearing C4b2a were incubated in the presence or absence of AChE as described in the text. At various time points, samples were removed and incubated with EDTA-chelated guinea pig serum to measure C4b2a activity. The decay rate of C4b2a was unaffected by the presence of AChE. Slope = −0.01 with AChE and −0.02 without AChE.

The first immunoprecipitation with anti-DAF (Lane A) removes a large amount of DAF from the lysate; however, by the final incubation with anti-DAF (Lane C), all DAF has been removed. Lanes B and D are normal rabbit serum (NRS) controls for the first and final precipitations, respectively. AChE was precipitated from the sample which had been previously cleared of DAF (Lane E). AChE appears in its dimeric form under the nonreducing conditions of this gel. AChE was also precipitated from the samples previously incubated with NRS (Lane G). Lanes F and H are immunoprecipitations using the P3 negative control after treatment with anti-DAF or NRS, respectively.
DISCUSSION

The abnormal erythrocytes in paroxysmal nocturnal hemoglobinuria are unusually susceptible to the hemolytic action of complement. This is due to an abnormality or abnormalities of the red cell membrane which results in a greater efficiency in the activation of complement on the membrane surface. The deficiency of decay-accelerating factor (DAF), a glycoprotein with a molecular weight of 70 Kd, appears to permit greater activation of C3 on the surface of the cell. However, other abnormalities must be present to account for the marked susceptibility to lysis of PNH III (the most abnormal) cells.

The first membrane abnormality described in PNH was the lack of the activity of the enzyme acetylcholinesterase. Recently, AChE itself has been shown to be absent from the membranes of PNH III erythrocytes. This enzyme has no known function on the cell and its absence or inhibition does not seem to affect the efficiency with which complement is activated. Acetylcholinesterase, like DAF, is about 70 Kd in molecular weight, leading to the suggestion that DAF and AChE activity were in fact resident on the same protein.

The present studies are clear in showing that this is not the case. Inhibition of one activity by antibody does not inhibit the other. The binding of antibody to one does not inhibit the binding of antibody to the other. Immunoprecipitation of one from the solubilized membrane proteins does not remove the other. Finally, inhibition of one (DAF) by antibody alters the effect of complement but inhibition of the other (AChE) does not.

This suggests that the membrane abnormalities in PNH must be complex. The defect is not likely to be due to gene deletion because the precursors of the abnormal erythrocytes appear to possess DAF activity. Rather, the underlying abnormality is probably an alteration in the posttranslational processing or interactions of the membrane proteins. It has been suggested that this might be an abnormality of glycosylation since in some instances the major glycoproteins of the red cell membrane are abnormal; it would be somewhat difficult to account for the total lack of the glycoproteins DAF and AChE from an abnormality in glycosylation unless the abnormality were to destabilize the protein in the membrane.

Whatever the fundamental abnormality is in PNH, it affects at least two distinct membrane proteins, acetylcholinesterase and decay-accelerating factor, of the erythrocytes of patients with the disease. The reason they are both lacking from these cells remains unknown.

ACKNOWLEDGMENT

We would like to thank Sharon Hoffman for technical assistance.

REFERENCES


15. Fraker PJ, Speck JC: Protein and cell iodinations with a sparingly soluble chloramide 1,3,4,5-tetrachloro-3,6-di-phenylglycuril. Biochem Biophys Res Commun 80:849, 1978


Structural and functional differences between decay-accelerating factor and red cell acetylcholinesterase [published erratum appears in Blood 1987 Jul;70(1):339]

J Sugarman, DV Devine and WF Rosse

Updated information and services can be found at:
http://www.bloodjournal.org/content/68/3/680.full.html

Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml