The Population of Paroxysmal Nocturnal Hemoglobinuria Neutrophils Deficient in Decay-Accelerating Factor Is Also Deficient in Alkaline Phosphatase

By Susan F. Burroughs, Dana V. Devine, Graeme Browne, and Manuel E. Kaplan

In patients with paroxysmal nocturnal hemoglobinuria (PNH) the RBCs, neutrophils (PMNs), monocytes, and platelets derived from the abnormal clone are deficient in the complement-regulatory protein decay-accelerating factor (DAF). RBC acetylcholinesterase (AChE) and leukocyte alkaline phosphatase (LAP) activities are also characteristically low. DAF, AChE, and LAP are known to be anchored within cell membranes to glycosphospholipid-containing phosphatidylinositol (PI). Because PNH progenitors contain DAF that appears to be lost with maturation, it has been proposed that this disorder results from abnormal tethering of these and possibly other proteins to membrane PI. We were puzzled, therefore, that our two PNH patients consistently had normal LAP levels. Consequently, we studied their isolated PMNs to compare DAF and LAP activities in individual cells. PMNs were separated by flow cytometry into DAF-positive and -negative populations by using rabbit anti-DAF antiserum and fluorescein-conjugated goat antirabbit IgG. In both patients the majority of PMNs were DAF deficient, and these cells contained very little alkaline phosphatase activity. In contrast, the smaller, DAF-positive cell populations were phosphatase replete. This is the first demonstration that abnormalities in DAF and LAP activity occur in the same PNH PMN population and strengthens the hypothesis that defective anchoring of proteins to membrane glycosphospholipid underlies the pathophysiology of this disorder.

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MATERIALS AND METHODS

Case reports. Patient W.W. is a 61-year-old white male who has a 41-year history of PNH. Because he was initially transfusion dependent and the diagnosis was unclear, he underwent splenectomy in 1949. Little improvement resulted, and the patient required intermittent transfusions until 1952. Thereafter, his clinical condition and blood counts gradually improved so that by 1958 he was no longer anemic and had less than 2% reticulocytes. His disease remained totally quiescent until April 1985. Since then there has been a progressive fall in the hemoglobin concentration (from 14.6 to 11.5 g/dL), and mild neutropaenia has developed; however, he remains asymptomatic with modest reticulocytosis (3.7% to 4.6%) and low LAP activity. LAP measurements performed on numerous occasions have ranged from 52 to 145 (normal, 30 to 100).

Patient S.Z. is an 80-year-old white male who presented in May 1986 with pancytopenia (hemoglobin, 6.7 gm/dL; WBC, 3,400/μL; platelet count, 29,000/μL); 4.6% reticulocytes, and a hypoplastic bone marrow. Sucrose hemolysis and Ham’s test results were positive. The LAP score was 148 and 216 on two separate occasions. Despite oral prednisone therapy (40 mg every other day), he requires red cell transfusions every 3 to 8 weeks.

Erythrocyte complement lysis sensitivity. Erythrocyte complement lysis sensitivity was measured by the method of Rosse and Dacie.

Neutrophil isolation. After informed consent, 25 mL heparinized blood was obtained from each patient and two normal controls. After dextran sedimentation for 40 to 50 minutes at 4°C, nonseminated cells were washed with phosphate-buffered saline (PBS) containing 10 mmol/L EDTA and centrifuged at 500 g for five minutes at 4°C. Contaminating erythrocytes were lysed by two brief exposures to hypotonic saline (0.2% NaCl) followed by restoration of isotonicity with 1.6% NaCl. After three washes with PBS-EDTA, leukocytes (>90% PMNs) were resuspended in 1 mL PBS-EDTA at a concentration of approximately 1 x 106 cells/mL.

Neutrophil labeling and sorting. Rabbit anti-DAF antiserum was prepared by the method of Nicholson-Weller et al. This antiserum specifically reacts with DAF in PMNs and platelets as determined by immunoblot analysis. Preimmune rabbit serum was used as a negative control. Fluorescein-conjugated (FC) goat anti-

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rabbit IgG was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). To 100 µL of a 1 × 10³ PMN/mL suspension in PBS-EDTA was added an equal volume of a 1:40 dilution of anti-DAF or control rabbit serum containing 1% bovine serum albumin. After a 45-minute incubation at 4°C, the cells were washed three times with PBS-EDTA and resuspended in 100 µL of this buffer. One hundred microliters of a 1:80 dilution of FC goat antirabbit IgG was then added and the mixture incubated for an additional 45 minutes at 4°C. The cells were again washed three times and resuspended at the original concentration in PBS-EDTA. Labeled PMNs were detected by using a sorted by a 440 flow cytometer equipped with Consort 40 computer (Becton Dickinson, Sunnyvale, CA).

LAP. Peripheral blood films and cytocentrifuged preparations of dextran-sedimented PMNs as well as the DAF-positive and -negative fractions thereof were fixed in 10% formalin in methanol and stained for phosphatase activity.18 Staining intensities (0 to 4+) of 100 consecutive PMNs were assessed.

RESULTS
The blood of patient W.W. was found to contain 85.5% PNH I RBCs (normal complement sensitivity) and 14.5% PNH III cells. Because patient S.Z. had received multiple transfusions before being studied, only 3.8% PNH III RBCs were detected in his peripheral blood, the remainder exhibited normal complement sensitivity. The LAP activities of the peripheral blood smears and isolated PMN populations are summarized in Table 1. In each control subject there was excellent agreement between LAP scores derived from the peripheral blood smear and the isolated (dextran-sedimented), unfractionated PMN preparation. In contrast, the isolated PMNs from both patients contained less phosphatase activity than was detected in their peripheral blood smears. The most likely explanations for this observation are (a) that many faintly stained, LAP-deficient PNH neutrophils were not discerned in the phosphatase-stained blood smears, which resulted in an overestimation of the patients' LAP scores, or (b) that the method used to isolate PMNs from peripheral blood, i.e., dextran sedimentation followed by hypotonic lysis of residual red cells, resulted in a selective loss of LAP-replete PNH neutrophils (in contrast to normal PMNs). Because the overall recovery of neutrophils from the blood of the patients and normal controls was not systematically studied, this possibility cannot be discarded.

When isolated PMNs from patients and control subjects were treated with nonimmune rabbit serum followed by FC antirabbit IgG and sorted, the homogenous nonfluorescent cell populations that resulted had LAP scores essentially identical to those of untreated cells. After anti-DAF treatment, control PMNs were uniformly DAF-positive, and their LAP content was little changed. In contrast, PNH PMNs were separable into distinct DAF-positive and -negative cell populations; in each case the smaller DAF-positive population exhibited normal phosphatase activity (Fig 1B), whereas the preponderant DAF-negative cell populations were markedly LAP deficient (Fig 1A).

DISCUSSION
A number of abnormalities have been recognized in PNH blood cells including deficiencies of the membrane proteins DAF (in PNH II and III RBCs, myeloid cells, and platelets),2,3,19-21 AChE (in PNH II and III RBCs),9 LAP (in PMNs),24 and HRF (in PNH III RBCs).30 DAF inhibits the assembly and facilitates the decay of the C3 convertases generated by both the classic (C4b2a) and the alternative (C3bBb3b) complement pathways.2,32 DAF deficiency appears to adequately explain the increased propensity of PNH RBCs to bind C3b, which facilitates C5 binding and activation of the terminal MAC.23 The deficiency in HRF, which normally inhibits insertion of C9 into cell membranes,24 is probably the major predisposing cause of the in vivo hemolysis.21

The pathophysiological relationship between PNH and abnormalities of RBC AChE and PMN alkaline phosphatase has, until very recently, defied explanation. Although the degree of AChE deficiency has been reported to correlate with PNH severity,24 inhibition of AChE activity of normal RBCs does not increase their complement sensitivity.9 Similarly, a correlation between LAP deficiency and PNH severity has been reported; however, this deficiency is not pathognomonic for PNH. It is now recognized that each of the membrane constituents deficient in PNH cells, with the possible exception of HRF, are tethered to a glycosphingolipid moiety containing PI.10-14 Thus, a PI-specific phospholipase C selectively cleaves DAF, AChE, and LAP from cell membranes.13,14 Because early progenitors of PNH RBCs contain DAF that appears to be lost as the cells mature,25 it was suggested that the pathophysiological basis for this disorder is an inability to properly tether these proteins to PI within cell membranes.

Studies have shown that DAF and AChE deficiencies coexist within PNH II and III RBCs.8 An analogous relationship between DAF and LAP in PNH PMNs has not been demonstrated. The present studies address this relationship. Our findings clearly indicate that the PMNs derived from the PNH clone, identified by being deficient in DAF, are also lacking in alkaline phosphatase activity (Table 1, Fig 1A); in contrast, the DAF-positive PMNs, which probably represent progeny of residual normal marrow elements, are LAP replete (Table 1, Fig 1B). Indeed, the presence of the latter cell population obscured the profound LAP deficiency present in the affected PMNs of our patients. Thus, our observations are completely consistent with the concept that defective anchoring of certain proteins to membrane glycosphingolipids underlies the pathogenesis of PNH.

### Table 1. Alkaline Phosphatase in DAF-Positive and -Negative Neutrophils of PNH Patients

<table>
<thead>
<tr>
<th>Subject</th>
<th>PB*</th>
<th>Unfractionated</th>
<th>LAP Scores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>DAF-Positive</td>
</tr>
<tr>
<td>W.W.</td>
<td>104</td>
<td>39</td>
<td>102 (25%) t</td>
</tr>
<tr>
<td>S.Z.</td>
<td>120</td>
<td>47</td>
<td>144 (40%) t</td>
</tr>
<tr>
<td>Control</td>
<td>73</td>
<td>58</td>
<td>67 (&lt;95%) ND</td>
</tr>
<tr>
<td>Control</td>
<td>129</td>
<td>110</td>
<td>75 (&lt;95%) ND</td>
</tr>
</tbody>
</table>

*Peripheral blood film (n = 30 to 100).
†Percentage of sorted cells.
‡Not done.

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Fig 1. Alkaline phosphatase staining of PNH neutrophils (patient W.W.). (A) DAF-negative cells. (B) DAF-positive cells.

REFERENCES


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